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Award Number: DAMD17-98-1-8586

TITLE: PTPμ Regulates Cell Adhesion and Signaling in Human Prostate Cancer Cells

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REPORT DATE: June 2002

TYPE OF REPORT: Annual, Phase II

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual, Phase II (1 Jun 00 - 31 May 02)

4. TITLE AND SUBTITLE
PTPμ Regulates Cell Adhesion and Signaling in Human Prostate Cancer Cells

5. FUNDING NUMBERS
DAMD17-98-1-8586

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We are investigating how cell adhesion-induced signals are transduced to negatively regulate cell growth and how it is altered in prostate cancer. Extracellular events that regulate cell growth are transmitted by changes in tyrosine phosphorylation, which is controlled by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Cancer causing genes encode PTKs that cause uncontrolled cell growth suggesting that PTPs play a role in negative growth regulation. Both cell adhesion molecules and tyrosine phosphorylation regulate contact inhibition of growth. Prostate cancer cells have defects in cell adhesion and contact inhibition. The receptor PTP, PTPμ, directly interacts with E-cadherin, the cell-cell adhesion molecule in prostate cells. Loss of components of the cadherin pathway has been observed in prostate cancer cells. We recently demonstrated that PTPμ is no longer expressed in prostate cancer cells. Re-expression of PTPμ restores adhesion mediated by PTPμ, E-cadherin and N-cadherin as well as negatively regulates cell growth. A detailed analysis of how PTPμ alters adhesion, cell growth and signal transduction is described and provides insights into both normal cell growth as well as malignant transformation in prostate cancer. Our results indicate that PTPμ is required for normal prostate cell adhesion and its loss plays a role in cancer progression.

14. SUBJECT TERMS
Tyrosine phosphorylation, Protein Tyrosine Phosphatases, Cadherins
Catenins, LNCaP

15. NUMBER OF PAGES
61

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TITLE: PTPμ regulates cell adhesion and signaling in human prostate cancer cells

PRINCIPAL INVESTIGATOR: Susann M. Brady-Kalnay

PHASE II TECHNICAL ABSTRACT:

Prostate cancer is the most common malignancy in men in the United States and the second leading cause of cancer mortality. The precise genetic pathway that leads to prostate cancer formation has not yet been elucidated but alterations in cell adhesion are often an important step towards deregulation of cellular proliferation and differentiation leading to invasion and metastasis. The major cell-cell adhesion molecules in differentiated epithelial cells are cadherins. Loss of components of the cadherin/catenin pathway has been observed in 50% or more of prostate cancer patients. Cadherins are localized at cellular communication sites called adherens junctions, which maintain the integrity of the epithelial sheet. The cytoplasmic domain of cadherins interacts with molecules termed catenins, which associate with the actin cytoskeleton. Studies have demonstrated reduced E-cadherin expression in a number of invasive and metastatic prostate tumors. In addition, reduction of E-cadherin protein levels and deletion of the α catenin gene have been observed in some prostate cancer cell lines. Many studies suggest that when any protein in the cadherin/catenin pathway was altered during malignant transformation it caused the same net cellular defect, it led to decreased adhesion, invasion and metastasis. Restoration of E-cadherin expression in prostate cancer cells has been shown to decrease invasion. We previously demonstrated that the protein tyrosine phosphatase (PTP), PTPμ, directly interacts with classical cadherins (such as E and N-cadherin). PTPμ is endogenously expressed in normal prostate cells. Surprisingly, we have found that the LNCaP prostate cancer cell line does not express PTPμ. LNCaP cells do express E-cadherin, N-cadherin and the catenins although the LNCaP cells are deficient in cadherin-dependent adhesion. A major goal during phase I was to add PTPμ to the LNCaP prostate cancer cells and determine how this affects adhesion, tyrosine phosphorylation, and cell growth.

During Phase I, we generated reagents to analyze how loss of PTPμ affects LNCaP cells. We generated retroviral constructs that express wild type and mutant forms of PTPμ with altered catalytic activity. These PTPμ-encoding retroviruses were used to infect LNCaP cells. Surprisingly, when either wild type or mutant forms of PTPμ were added to LNCaP cells, they restored adhesion to PTPμ, E-cadherin and N-cadherin. These studies suggested that phosphatase activity of PTPμ was not required to restore cadherin-dependent adhesion. We then generated another mutant form that contained the entire extracellular and transmembrane domains of PTPμ but lacked the phosphatase domains (PTPμ-extra). PTPμ-extra did induce adhesion to PTPμ as expected. Importantly, PTPμ-extra did not restore adhesion to E-cadherin. These data indicate that the presence of the phosphatase domains is required to restore E-cadherin-dependent adhesion. Our working hypothesis is that the phosphatase domains may be recruiting proteins to the E-cadherin complex that regulate adhesion. Interestingly, re-expression of wild type PTPμ negatively regulated growth. However, none of the mutant forms of PTPμ affected cell growth. These data indicate that PTPμ phosphatase activity is required to negatively regulate cell growth.

Phase II Objective: We were planning to extend the studies carried out in phase I. We found that re-expression of PTPμ restored cadherin-dependent adhesion and negative growth regulation. These data suggest that PTPμ may mediate contact inhibition of growth. We were now interested in elucidating the molecular mechanisms by which PTPμ induced changes in these cellular phenomena. Specifically, we were testing the following hypothesis in phase II: Alterations in PTPμ expression in prostate cancer result in changes in specific cell-cell adhesion-induced signal transduction pathways that abrogate contact inhibition of growth. The study of the coordinated regulation of adhesion and tyrosine phosphorylation by PTPμ was important for understanding both the loss of adhesion and loss of growth control seen upon malignant transformation in prostate cancer.

ACRONYMS:

PTP, protein tyrosine phosphatase

RPTP, receptor protein tyrosine phosphatase

PTK, protein tyrosine kinase

RPTK, receptor protein tyrosine kinase

FNIII, fibronectin type III repeat

PKC, protein kinase C

RACK1, receptor for activated protein kinase C

PI3K, phosphatidyl inositol 3 kinase

PH domains, pleckstrin homology domains

Cdk, cyclin-dependent kinase

RSV, Rous sarcoma virus

PTPμ mutants: C-S, PTPμC1095S mutant
D-A, PTPμ D1063A mutant
R-M, PTPμR1101M mutant
PTPμ-extra, construct containing the extracellular-, transmembrane- and 55 amino acids of the
intracellular domains of PTPμ

GFP, green fluorescence protein

BSA, bovine serum albumin

PBS, phosphate buffered saline

ORIGINAL STATEMENT OF WORK

SPECIFIC AIMS:

I. Determine the mechanism by which PTPμ restored E-cadherin-dependent adhesion

A. Re-express mutant forms of PTPμ that restored E-cadherin-dependent adhesion in LNCaP cells and determine if there were changes in any PTPμ associated proteins

1) Determine if the PTPμ mutants associate with RACK1 and if the association between RACK1 and PTPμ recruits activated PKC or other signaling molecules to the cadherin complex. (four months)

2) Determine if PTPμ displaces the src PTK from the cadherin-complex in LNCaP cells and if this alters the tyrosine phosphorylation state of proteins in the complex. (three months)

B. Utilize inhibitors of PKC and src to demonstrate a functional role for these signaling proteins in the restoration of adhesion induced by PTPμ.

1) PKC stimulation or inhibition will be used to determine if PKC activation is required for restoration of E-cadherin-dependent adhesion. (four months)

2) The src tyrosine kinase inhibitor PP1 will be used to determine if src inhibition in LNCaP cells restores E-cadherin-dependent adhesion. (three months)

II. Elucidate the signal transduction pathway that PTPμ utilizes to negatively regulate cell growth

A. Re-express wild type PTPμ in LNCaP cells and determine the expression and/or the activation state of signaling proteins in the PI3K pathway.

1) Analyze cell cycle changes when PTPμ is re-expressed in LNCaP cells (two months)

2) Examine the activation state of proteins in the PI3K pathway (four months)

B. Utilize PI3K inhibitors to determine if the PI3K pathway is regulated by wild type PTPμ. (three months)

INTRODUCTION:

Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths in men in the United States. The mechanism of cancer progression is not well understood but alterations in cell adhesion are often an important step towards deregulation of cellular proliferation and differentiation leading to invasion and metastasis. The major cell-cell adhesion molecules in differentiated epithelial cells are cadherins. Cadherins are localized at cellular communication sites called adherens junctions and have been implicated in the regulation of adhesion, motility and cell growth, but the mechanism or signal transduction pathways are not presently understood [1-3]. The cytoplasmic domain of cadherins interacts with molecules termed catenins that associate with actin [1]. The catenins include α, ß, γ/plakoglobin and p120 (some components shown in Fig.1). α catenin is homologous to the cytoskeletal-associated protein vinculin and binds actin directly. ß catenin (also called *armadillo*) is an "arm repeat" protein and is homologous to plakoglobin and p120. γ catenin and plakoglobin are identical [4, 5]. Other cadherin-associated proteins include APC (adenomatous polyposis coli-a catenin-associated protein), a number of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [6-8]. Normal PTKs and their oncogenic counterparts associate with cadherins including *met*, *src*, c-erbB2 and the EGF receptor [9]. Activation of tyrosine kinases results in a loss of the differentiated phenotype, decreased cell adhesion and loss of adherens junctions. Alterations in any one of the components of the cadherin complex may cause changes in cell-cell adhesion leading to the malignant progression.

Cadherins and their associated proteins have proven to be either tumor suppressor genes or oncogenes indicating this "pathway" is crucial for normal prostate epithelial cell growth [3]. The exact genetic pathway that leads to prostate cancer formation has not yet been elucidated. However, there are a number of mutations in prostate tumors that are relevant to this report because they include members of the cadherin/catenin complex [10-13]. First, mutations on chromosome 5q cluster around the APC and α catenin genes [14]. The PC3 prostate cancer cell line has deletions in the coding region of the α catenin gene [15, 16]. Second, deletions of 16q are commonly observed in prostate cancer and span the region containing the E-cadherin gene [14, 17, 18]. Third, mutations in β catenin have been observed in approximately 5% of prostate cancer patients [19]. Fourth, 17q loss of heterozygosity is observed near the BRCA1 locus which is adjacent to the γ catenin/plakoglobin gene [14, 20]. Finally, the c-erbB2 [21] and c-*met* [22] receptor tyrosine kinases are implicated in prostate cancer progression [14]. Loss of components of the cadherin/catenin pathway result in uncontrolled cell growth, invasion or metastasis.

Tyrosine phosphorylation is involved in the regulation of a diverse set of cellular behaviors including growth, differentiation, and migration. Often tyrosine phosphorylation is a molecular switch, turning on or off various protein-protein interactions. Phosphotyrosine levels are controlled by the competing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). A diverse family of receptor-like (RPTPs) and nontransmembrane PTPs have been identified and characterized [23, 24]. Some of the RPTPs have structural homology to cell-cell adhesion molecules such as the neural cell adhesion molecule, N-CAM (Fig.1). N-CAM contains multiple Ig domains and fibronectin type-III (FNIII) repeats in its extracellular segment and functions as a homophilic cell-cell adhesion molecule [25, 26]. The RPTPs have cell adhesion molecule-like extracellular segments with intracellular segments that possess tyrosine phosphatase activity. Presumably the RPTPs are directly transducing intracellular signals in response to cell adhesion.

This study focuses on the RPTP, PTPμ (Fig. 1). The RPTPs have one or two conserved catalytic domains in their intracellular segments while their extracellular segments are quite diverse [6-8]. PTPμ contains a MAM domain, an Ig domain and four FNIII repeats in its extracellular segment, which suggested that it may function in cell-cell adhesion. In fact, we demonstrated that PTPμ participates in homophilic binding interactions [27] via its immunoglobulin domain [28]. The PTPμ

adhesion molecule was also shown to promote neurite outgrowth from retinal neurons *in vitro* [29]. The juxtamembrane domain of PTPμ contains a region of homology to the conserved intracellular domain of the cadherins [30]. Since the cytoplasmic domain of cadherins interacts with catenins, this led us to speculate that PTPμ may also interact with catenins [30].

To test whether PTPμ could associate with cadherins or catenins we used cells and tissues where PTPμ is endogenously expressed including heart, lung, and brain tissues and two immortalized cell lines: MvLu (derived from lung) and WC5 cells (derived from brain). Immunoprecipitations from all of these cell lines or tissues demonstrated that PTPμ associates with a complex containing cadherins, α catenin and ß catenin [31, 32]. Specifically, PTPμ interacts with N-cadherin, E-cadherin and cadherin-4 (also called R-cadherin) [32]. We demonstrated that the intracellular segment of PTPμ binds directly to the intracellular domain of E-cadherin. Our results were later confirmed by another group [33]. We then used a series of WC5 cell lines, which express PTPμ endogenously and ectopically expressed mutant forms of E-cadherin that lack various portions of the cytoplasmic segment. The WC5 cell studies indicated that the C-terminal 38 amino acids were required for the interaction with PTPμ. We had clearly established an interaction between PTPμ and cadherins by immunoprecipitation, however, we have recently demonstrated that PTPμ function is required for N-cadherin-dependent axonal migration (regulation of neurite outgrowth) [29]. Subsequently, other PTPs were shown to interact with cadherins and catenins [34-38]. Together, these data suggest it is likely that regulation of cadherin/catenin complex by PTPs will be an important mechanism of control in many cell types and tissues of the body.

A number of cytoplasmic and receptor protein tyrosine kinases (PTKs) including *src*, EGF receptor and *met*, (scatter factor receptor), phosphorylate components of the cadherin/catenin complex [39-41]. Tyrosine phosphorylation of components of the cadherin/catenin complex suppresses cadherin-mediated adhesion and destabilizes adherens junctions [1, 6]. We tested whether tyrosine phosphorylation affects PTPμ/cadherin interactions. We used the WC5 cell line, which is transformed with a temperature-sensitive form of the Rous Sarcoma Virus (RSV) [32]. The mutant RSV is temperature sensitive for pp60*src* tyrosine kinase activity. The data suggested that increased tyrosine phosphorylation induced by *src* results in decreased association between PTPμ and E-cadherin. This decreased association correlates with increased tyrosine phosphorylation of E-cadherin. The association of the cadherins with both tyrosine kinases and phosphatases indicates a critical role for dynamic phosphorylation in cadherin function, however, the precise mechanism is unknown.

We demonstrated that the phosphatase, PTPμ, directly mediates cell adhesion and interacts with cadherins, and that this complex is regulated by tyrosine phosphorylation [27, 31, 32]. It is clear that PTPμ mediates cell adhesion but it is not known whether this affects enzymatic activity. However, it is likely that PTPμ regulates both cell-cell adhesion and signaling. It was important to identify the mechanism by which PTPμ alters signaling and the crucial substrates involved. To investigate the role of PTPμ in cadherin function, we identified a system in which we could add wild type and mutant forms of PTPμ and look at effects on cadherin-dependent adhesion. PTPμ is endogenously expressed in normal prostate cells (Fig. 2). Surprisingly, we have found that the LNCaP prostate cancer cell line does not express PTPμ (Fig. 2) suggesting that loss of PTPμ contributes to tumorigenesis. The LNCaP cell line is used as a model system for prostate cancer because it is responsive to androgen and expresses prostate specific markers such as prostate specific antigen and prostatic acid phosphatase [42]. LNCaP cells do express normal levels of cadherins and a number of their associated proteins (Fig. 3). We have found that LNCaP cells were deficient in cadherin-dependent adhesion (Fig. 4). A major goal of this study was to add normal and mutant forms of PTPμ, using a retroviral expression system, to the LNCaP cells and determine how this affects adhesion, tyrosine phosphorylation, growth and tumorigenicity of the cells. As described below, our data suggest that there is a dramatic effect on adhesion when PTPμ is re-expressed in LNCaP cells (Fig. 11). The restoration of adhesion was independent of phosphatase

activity (Fig. 11). Interestingly, re-expression of wild type PTPμ negatively regulated growth (Fig. 21). However, none of the mutant forms of PTPμ affected cell growth. This data indicates that PTPμ phosphatase activity is required to negatively regulate cell growth. These data suggest that PTPμ may mediate contact inhibition of growth.

BODY:

PHASE II HYPOTHESIS/PURPOSE: **We were testing the hypothesis that changes in PTPμ expression during prostate cancer progression modulate cell-cell adhesion and signaling that abrogates contact inhibition of growth.** Growth inhibitory signals are transmitted by cell-cell contact as normal cells reach confluence. In cancer, such "contact inhibition of growth" is lost. The concept of contact inhibition of growth has been a popular concept for decades but the molecular mechanisms are still not understood. The significance of the proposed research is to investigate how cell-cell adhesion events trigger signals that ultimately regulate growth in prostate cancer. Since most signals regulate cell growth via tyrosine phosphorylation, it is not surprising that a number of protein tyrosine kinases (PTKs) are oncogenes. Therefore, PTPs may be negatively regulating growth by dephosphorylating key cellular proteins. A number of studies using generic inhibitors have linked PTPs to contact inhibition of growth [6]. The PTPs that mediate contact inhibition of growth have yet to be identified, however receptor-type phosphatases (RPTPs) are attractive candidates because they may transduce signals directly in response to adhesion. In fact, our data suggests that re-expression of wild type PTPμ inhibited growth, and phosphatase activity was required. The ability of PTPμ to negatively regulate growth suggests it may be involved in contact inhibition of growth. Loss of contact inhibition of growth and adhesion-based signal transduction is crucial for the development of the transformed state. **During Phase II, we investigated the molecular mechanisms by which PTPμ restores adhesion and negatively regulates cell growth.**

PHASE II SPECIFIC AIMS:

I. Determine the mechanism by which PTPμ restores E-cadherin-dependent adhesion
To investigate the role of PTPμ in cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cells, which do not express endogenous PTPμ (Fig. 2). Although LNCaP cells express E-cadherin and N-cadherin as well as α-, ß- and γ-catenin and p120, we found that the cells were deficient in cadherin-mediated adhesion (Fig. 9). Re-expression of wild type PTPμ restored adhesion, demonstrating a functional role for PTPμ in cadherin-mediated adhesion (Fig. 11). The fact that the re-expression of the catalytically inactive mutants also restored cadherin-mediated adhesion indicates that PTPμ exerts effects on the cadherin-catenin complex that are independent of its catalytic activity (Fig. 11). In this aim, we tested two hypotheses pertaining to the molecular mechanism by which PTPμ restores cadherin-dependent adhesion. Our first hypothesis was that the PTPμ intracellular domain may be recruiting proteins to the cadherin complex that are responsible for restoring adhesion (Aim I-A-1). Our second hypothesis is that even catalytically inactive PTPμ may be indirectly altering the tyrosine phosphorylation state of a protein in the cadherin complex by changing the ability of the *src* tyrosine kinase to interact with the complex (Aim I-A-2).

The first hypothesis is based on the idea that the intracellular domain of PTPμ may be recruiting proteins to the cadherin-complex via protein-protein interactions. Others have reported that the intracellular domains of RPTPs mediate protein-protein interactions that are phosphotyrosine-independent [43-45]. The fact that PTPμ, regardless of its catalytic activity, could restore cadherin-mediated adhesion suggests that part of its role in the cadherin complex is to recruit other signaling molecules that may be needed for functional adhesion. The importance of the intracellular domains of PTPμ is clearly demonstrated by the finding that LNCaP adhesion to E-cadherin was not

restored by the expression of PTPμ-extra, in which the phosphatase domains of PTPμ had been deleted (Fig.11).

A. Re-express mutant forms of PTPμ that restore E-cadherin-dependent adhesion in LNCaP cells and determine if there were changes in any PTPμ associated proteins

1. Determine if the PTPμ mutants associate with RACK1 and if the association between RACK1 and PTPμ recruits activated PKC or other signaling molecules to the cadherin complex.

Our data suggest an interesting relationship exists between PKC signal transduction mechanisms (RACK1) and the PTPμ cell-cell adhesion molecule. Protein kinase C is an important signaling molecules which becomes activated in response to cell stimulation, such as growth factor stimulation of receptor tyrosine kinases via phospholipase C gamma (PLCγ). Activated PKC is then responsible for stimulating other intracellular targets including Ca^{++} mobilization. We have determined that LNCaP cells express RACK1 (Fig.14). LNCaP cells express the PKCδ isoform (Fig. 14). The association of RACK1 and PTPμ may bring activated PKC to the membrane. PKC may regulate E-cadherin-dependent adhesion. Adherens junctions serve to anchor the actin cytoskeleton at regions of cell-cell contact. Investigators have postulated that the cellular reorganization occurring in the formation of adherens junctions is induced by PKC activation and that PKC may regulate cadherin-dependent adhesion [46].

Methods: We investigated whether the composition of the cadherin/catenin complex and the PTPμ complex were altered in LNCap cells by re-expression of wild type or mutant forms of PTPμ. Specifically, we performed immunoprecipitation/ immunoblot analyses as previously described [31]. We immunoprecipitated PTPμ, cadherins/catenins, RACK1 and PKCδ. We immunoblotted those immunoprecipitates with antibodies to PTPμ, RACK1 and PKCδ. Monoclonal antibodies against PTPμ have been described [27, 28]. We also determined if these proteins co-localize by immunocytochemistry. Monoclonal antibodies to RACK1, and PKCδ were purchased from Transduction Labs, Cell Signaling and Calbiochem.

2. Determine if PTPμ displaces the src PTK from the cadherin-complex in LNCaP cells and if this alters the tyrosine phosphorylation state of proteins in the cadherin/catenin complex.

We have previously shown that PTPμ and *src* differentially regulate the cadherin phosphorylation state or composition of the complex [32] but the precise mechanism is unclear. The C-terminal 38AA of the cadherins are crucial for cadherin-dependent adhesion and cytoskeletal association [47, 48]. The C-terminal 38AA contain the binding site for both β catenin and PTPμ. Our preliminary experiments indicate that the major site of tyrosine phosphorylation by *src* is in the C-terminal 38AA of E-cadherin. We analyzed the effect of tyrosine phosphorylation on the composition of the PTPμ/cadherin complex and our data suggest that increased tyrosine phosphorylation of E-cadherin results in decreased association with PTPμ [32].

Methods: We investigated whether the composition of the PTPμ complex or activation state of potential downstream components of a signaling pathway were altered in LNCaP cells following re-expression of wild type or mutant forms of PTPμ. Specifically, we performed immunoprecipitation/immunoblot analyses. We immunoprecipitated PTPμ, cadherins/catenins, RACK1 and *src*. Multiple *src* antibodies are available that detect the protein itself or specific activation states (phospho-specific antibodies from Biosource Inc and Cell Signaling). Re-expression of PTPμ may indirectly alter the phosphorylation state of these proteins. The tyrosine phosphorylation of the proteins in PTP/cadherin complex was compared by immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies. We also determined if these proteins co-localize by immunocytochemistry.

B. Utilize inhibitors of PKC and *src* to demonstrate a functional role for these signaling proteins in the restoration of cadherin-dependent adhesion.

1. PKC stimulation or inhibition was used to determine if PKC activation is required for restoration of E-cadherin-dependent adhesion.

PKC signaling may be downstream of PTPμ based on the interaction between PTPμ and RACK1. Therefore, activation of PKC may be sufficient to restore cadherin-dependent adhesion without re-expression of PTPμ. This hypothesis was tested by stimulating PKC and analyzing cadherin-dependent adhesion in LNCaP cells. Treatment with phorbol esters (PMA), known tumor promoters, can stimulate PKC [49]. PKC activation of LNCaP cells was performed by adding 10nM PMA (Calbiochem) for 15min. at 37°C before testing E-cadherin-dependent adhesion. We tested the involvement of PKC in PTPμ-dependent signaling by re-expressing PTPμ in LNCaP cells then treating the cells with a PKC inhibitor (GF109203X-Sigma) and determining if this blocks the restoration of E-cadherin-dependent adhesion induced by PTPμ. PKC inhibition in LNCaP cells was done by adding 6nM GF109203X (Sigma) for 15min. before testing E-cadherin-dependent adhesion. In both cases, vehicle only (DMSO) was added as a negative control.

2. The src tyrosine kinase inhibitor PP1 was used to determine if src inhibition of LNCaP cells restores E-cadherin-dependent adhesion.

Activation of the *src* PTK is known to affect cadherin-dependent adhesion. We determined if inhibiting *src* tyrosine kinase activity restores E-cadherin-dependent adhesion even in the absence of PTPμ. *src* inhibition was done by adding 300nM PP1 for 15min. before testing E-cadherin-dependent adhesion. We also analyzed the state of *src* activation in LNCaP cells that re-express wild type or mutant forms of PTPμ. To determine if *src* was activated, we used phospho-specific antibodies to *src* (Biosource Inc and Cell Signaling). Changes in the activation state of *src* when PTPμ is re-expressed would suggest that *src* and PTPμ may be regulating the same pathway.

Results from Aim 1:

The major finding from the experiments outlined above were published recently [50].

Changes in PTPμ expression in prostate cancer

To determine if loss of PTPμ occurs in prostate cancer, we performed immunoblotting experiments. PTPμ expression was compared in prostate carcinoma cell lines and normal prostate cells (Clonetics Inc.). Normal prostate expresses full length PTPμ (200kDa) and the proteolytically processed fragment (100kDa). Changes in PTPμ expression were only observed in LNCaP and CWRU xenograft prostate cancer cells (Fig.2). The xenograft tumor cell system developed at CWRU by Dr. Tom Pretlow is serially passaged primary human prostate tumor cells [51]. We observed no PTPμ expression in both the androgen-dependent (CWR22) and relapsed (androgen-insensitive) cell lines (CWR22R). Therefore, changes in PTPμ expression may be relevant to a subset of prostate cancers.

The LNCaP cell line is a good model system for prostate cancer [42] because it is androgen responsive and expresses prostate specific genes such as PSA and prostatic acid phosphatase. LNCaP cells were previously shown to have the normal complement of both E-cadherin and α catenin (see also Fig. 3). In addition, the cadherin complex contained α/ß and γ catenin. We also observed normal expression and interaction of cadherins and catenins (Figs. 3 &12). However, LNCaP cells are defective in cadherin-dependent adhesion (Fig.4).

Re-expression of PTPμ

The receptor tyrosine phosphatase PTPμ has previously been shown to interact with E-cadherin in a variety of tissues by immunoprecipitation [31-33]. To investigate whether PTPμ plays a functional role in E-cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cell line [42]. Unlike normal prostate epithelial cells (NPr), these cells do not express PTPμ (Fig. 6A, VEC). To re-express PTPμ in LNCaP cells, we generated a tetracycline-regulatable retrovirus encoding the PTPμ cDNA sequence tagged with the green fluorescence protein (PTPμ-GFP) [29] (Fig. 5). Using this retrovirus, we re-expressed wild type PTPμ (PTPμWT) in the LNCaP cells. Five days after retroviral infection, the cells were analyzed for expression of PTPμWT-GFP by immunoblot and by fluorescence microscopy. Immunoblot analysis showed that LNCaP cells infected with retrovirus containing an empty vector do not express PTPμ (Fig. 6A, VEC). Cells infected with retrovirus containing PTPμWT (Fig 6A, WT) expressed both the full-length protein (200 kDa) as well as the proteolytically processed forms (~100 kDa) [28]. Due to the GFP-tag, both the full length- and the proteolytically processed forms of the re-expressed PTPμWT migrated at a higher molecular weight than the PTPμ expressed in normal prostate cells (Fig.6A, NPr). The retroviral system we used is a tet-off system, in the presence of tetracycline the gene is not expressed. The re-expression of PTPμWT was inhibited by treating the cells with tetracycline (Fig 6A, WT+T). Fluorescence microscopy revealed that between 70-90 % of the LNCaP cells expressed PTPμWT, and that PTPμ was primarily localized to the plasma membrane as expected (Fig. 7C and D). This expression was repressed when the cells were grown in the presence of 4 μg/ml of tetracycline (Fig. 7E and F). Control cells infected with a virus containing an empty vector did not show any fluorescence (Fig. 7A and B).

To assess the functional role of PTPμ catalytic activity in the regulation of E-cadherin-mediated adhesion, we have generated tetracycline-repressible retrovirus encoding a mutant form of PTPμ-GFP containing a single amino acid mutation in the catalytic site (Fig. 5) [29]. Mutation of the conserved cysteine residue PTPμC1095S (C-S) results in a catalytically inactive enzyme. Immunoblot analysis showed that the C-S mutant was expressed at a similar level to PTPμWT in LNCaP cells (Fig. 6A, C-S). Fluorescence microscopy confirmed that infection with the C-S mutant (Fig. 7G and H) resulted in expression at the plasma membrane at a similar level as PTPμWT, demonstrating that the expression and subcellular localization are not affected by the loss of catalytic activity.

Re-expression of PTPμ enhanced calcium-dependent aggregation of LNCaP cells

To investigate whether PTPμ plays a role in E-cadherin-mediated adhesion in LNCaP cells, we trypsinized the cells in the presence of $CaCl_2$ to selectively preserve the cadherins [52]. This assay only measures calcium-dependent aggregation predominantly mediated by the cadherins [52]. The cells were then allowed to aggregate for one hour. LNCaP cells infected with an empty vector weakly aggregated (Fig. 8). Re-expression of PTPμWT increased the aggregation 3 fold as did expression of the C-S mutant (Fig. 8). The increased aggregation was only partly dependent on E-cadherin function, since the presence of an E-cadherin function-blocking antibody did not completely reduce the aggregation induced by re-expression of PTPμ (Fig. 8). However, the increased aggregation was Ca^{2+}-dependent, since the presence of EDTA reduced the aggregation to a level below that seen in cells infected with an empty vector (Fig. 8). The residual Ca^{2+}-dependent adhesion is at least due in part to the fact that LNCaP cells express N-cadherin (data not shown). Taken together, these findings demonstrate that re-expression of PTPμ in LNCaP cells induced Ca^{2+}-dependent aggregation that is partly due to E-cadherin-dependent cell-cell adhesion. Thus, aggregation assays were not ideal to specifically study E-cadherin-dependent adhesion in LNCaP cells. Therefore, we utilized an *in vitro* adhesion assay that measures specific binding to a given adhesion molecule which is similar to our previously published assay [27].

Re-expression of PTPμ induced adhesion to purified PTPμ

To study the specific interactions between cell-cell adhesion molecules in LNCaP cells, we developed an *in vitro* adhesion assay where purified, recombinant proteins were immobilized on nitrocellulose-coated coverslips. Basically, three spots of protein (Laminin, E-cadherin and PTPμ) were added to each nitrocellulose-coated coverslip. The field shown in each panel represents virtually the entire spot for a given adhesion molecule. PTPμ has been shown to mediate cell-cell adhesion via homophilic binding [27, 53]. To verify that the re-expressed forms of PTPμ were able to mediate homophilic binding in LNCaP cells, we investigated the adhesion of LNCaP cells to purified recombinant PTPμ that was immobilized on nitrocellulose-coated coverslips. As expected, cells infected with an empty vector did not adhere to PTPμ (Fig.11A) since these cells do not express PTPμ. Re-expression of PTPμWT induced LNCaP adhesion to purified PTPμ (Fig. 11D), as did re-expression of the C-S mutant (Fig. 11G). Quantitation of the adhesion assays (N=6) showed that the number of cells that adhered to purified PTPμ was significantly higher for cells infected with both the WT and the C-S mutant form of PTPμ as compared to cells infected with vector only [50]. However, there was no difference between cells expressing PTPμWT compared to the C-S mutant in their ability to adhere to purified PTPμ. To ensure the specificity of the adhesion assay, we repeated the experiments in the presence of function-blocking antibodies to either PTPμ or E-cadherin. The presence of an antibody to the extracellular domains of PTPμ specifically inhibited the adhesion to recombinant PTPμ of LNCaP cells re-expressing PTPμWT (9, WT+ PTPμ Ab), or cells expressing the C-S mutant (Fig. 9, C-S+PTPμ Ab). As expected, the presence of the E-cadherin antibody had no effect on adhesion to PTPμ (Fig. 9, WT+E-cad. Ab, C-S+E-cad. Ab). Taken together, these data confirm that the re-expressed PTPμ is present at the cell surface and capable of mediating homophilic binding. In addition, PTPμ phosphatase activity is not necessary for PTPμ-dependent adhesion to occur as previously demonstrated [27].

As an internal control in each experiment, cells were allowed to adhere to Laminin. Adhesion to extracellular matrix proteins such as Laminin is mediated through integrin receptors. Since there is no evidence indicating that PTPμ regulates integrin function, LNCaP adhesion to Laminin should not be affected by the re-expression of PTPμ. As expected, LNCaP cells infected with an empty vector adhered to Laminin (Fig. 11B), and this adhesion was not significantly affected by re-expression of either WT (Fig. 11E) or C-S mutant forms of PTPμ (Fig. 11H). None of the retrovirally-infected cells adhered to nitrocellulose coated with BSA only (data not shown).

Re-expression of PTPμ restores E-cadherin-mediated adhesion

To study the role of PTPμ in the regulation of E-cadherin-mediated adhesion in LNCaP cells, we immobilized purified recombinant E-cadherin on the nitrocellulose-coated coverslips. Despite the fact that these cells express E-cadherin as well as α-, ß- and γ- catenin and p120 (Figs. 3 & 12), LNCaP cells infected with an empty vector did not adhere to E-cadherin (Fig. 11C). Re-expression of PTPμWT restored the ability of LNCaP cells to adhere to E-cadherin (Fig. 11F). Quantitation of the adhesion assays show that the number of cells infected with PTPμWT that adhered to E-cadherin was significantly higher than the number of cells infected with vector only [50]. These data show that expression of PTPμ is necessary for E-cadherin-mediated adhesion in LNCaP cells.

Since tyrosine phosphorylation has been reported to negatively regulate cadherin-mediated adhesion, we investigated whether PTPμ restored E-cadherin-mediated adhesion by dephosphorylating key components of the cadherin-catenin complex. To do this, we repeated the adhesion assays with cells expressing the C-S mutant form of PTPμ. Expression of the C-S mutant restored E-cadherin-mediated adhesion (Fig. 11I). Re-expression of WT or the C-S mutant form of PTPμ induced a significant increase in adhesion to E-cadherin as compared to LNCaP cells infected with an empty vector. In contrast, there was no difference in adhesion between cells infected with PTPμWT compared to cells infected with the C-S mutant [50]. In control

experiments, the adhesion to E-cadherin was totally blocked by a function-blocking antibody to E-cadherin (Fig. 10, WT+E-cad. Ab, and C-S+E-cad. Ab, respectively). In contrast, the PTPμ antibody did not affect the adhesion to E-cadherin induced by the re-expression of PTPμWT (Fig. 10, WT+PTPμ Ab) or by the expression of the C-S mutant (Fig. 10, C-S+PTPμ Ab) as expected. E-cadherin-mediated adhesion in this assay is Ca^{2+}-dependent, addition of 5 mM EDTA abolished adhesion to E-cadherin (Fig. 10, WT+EDTA, and C-S+EDTA, respectively). However, the presence of EDTA did not affect the adhesion to PTPμ, which is calcium-independent, of cells either re-expressing PTPμ-WT (Fig. 9, WT+EDTA) or expressing the C-S mutant (Fig. 9, C-S+EDTA). Taken together, these data indicate that although the presence of the PTPμ protein is required for E-cadherin-mediated adhesion in LNCaP cells it does not require PTPμ catalytic activity.

It is possible that the PTPμ intracellular domain may recruit other proteins that aid in restoring E-cadherin-mediated adhesion. To determine whether the intracellular PTP domains of PTPμ were required to affect E-cadherin-dependent adhesion, we constructed a retrovirus encoding the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (Fig. 5) (PTPμ-extra) [27]. Western blot analysis confirmed that this construct was expressed in LNCaP cells (Fig. 6B, Extra). The cytoplasmic domain of PTPμ is known to bind to E-cadherin [31]. Immunoprecipitation experiments confirmed that PTPμ-extra does not associate with E-cadherin (data not shown). Expression of PTPμ-extra induced LNCaP adhesion to purified recombinant PTPμ (Fig. 11J; Table I), confirming that the intracellular domains are not required for PTPμ to mediate homophilic binding [27]. In addition, adhesion to PTPμ was blocked by an antibody to PTPμ (Fig. 9, Extra+PTPμ Ab). The antibody to E-cadherin and 5mM EDTA had no major effect on the adhesion to PTPμ (Fig. 9, Extra+E-cad. Ab and Extra+EDTA, respectively) as expected. However, PTPμ-extra did not restore LNCaP adhesion to recombinant E-cadherin (Fig. 11L), demonstrating that the intracellular domains of PTPμ are necessary for restoring E-cadherin-mediated adhesion. Since LNCaP cells expressing PTPμ-extra did not adhere to E-cadherin (Fig. 11L; Fig. 10B, Extra), the presence of either the PTPμ antibody, the E-cadherin antibody or 5 mM EDTA had no effect on adhesion to E-cadherin (Fig. 10, Extra + PTPμ Ab, Extra + E-cad. Ab, and Extra + EDTA, respectively). As expected, the expression of PTPμ extra did not affect LNCaP adhesion to Laminin (Fig. 11K). Together, these results suggest that PTPμ-extra is expressed at the cell surface and capable of inducing adhesion to PTPμ but not restoring E-cadherin-dependent adhesion.

Similar to the results shown in Fig. 4, LNCaP cells expressing an empty vector did not adhere to either PTPμ or E-cadherin (Fig. 9, VEC, and Fig. 10, VEC, respectively), and this was not altered by the presence of either the PTPμ antibody, the E-cadherin antibody or 5 mM EDTA (data not shown). Taken together, these experiments demonstrate that this *in vitro* adhesion assay can be used to study specific binding to cell-cell adhesion molecules.

Expression of cadherins and catenins

Cadherin-mediated cell-cell adhesion is dependent on the expression of both cadherins and catenins. Immunoblot analysis demonstrated that LNCaP cells expressed E-cadherin as well as α, ß and γ-catenin and p120 (Fig. 3). This is in accordance with normal prostate epithelial cells, which were found to express similar amounts of E-cadherin as well as α, ß and γ-catenin (Fig.3). Infection of LNCaP cells with an empty vector, PTPμWT or the C-S mutant form of PTPμ did not alter the expression of any of the proteins in the cadherin/catenin complex (Fig. 12). It is possible that re-expression of wild type or mutant forms of PTPμ may alter the subcellular localization of the proteins in the cadherin/catenin complex, thereby altering the function of the complex. To address this question, we performed immunocytochemical analysis on LNCaP cells using antibodies to E-cadherin as well as α, ß and γ-catenin and p120. However, re-expression of either PTPμWT or the

C-S mutant did not significantly alter the subcellular localization of any of the proteins examined (data not shown).

PTPμ does not alter the association of α-, ß-, γ-catenin or p120 to E-cadherin

To examine the possibility that the presence of PTPμ affects the binding of α-, ß-, γ-catenin or p120 to E-cadherin, we immunoprecipitated E-cadherin from cells infected with an empty vector (VEC), PTPμWT (WT), C-S or PTPμ-extra (Extra). As shown in Fig. 12, the immunoprecipitates from cells infected with an empty vector, PTPμWT as well as the C-S and PTPμ-extra contained equal amounts of E-cadherin. The immunoblot was stripped and reprobed with antibodies to the catenins. Immunoprecipitates from cells infected with an empty vector contained α-, β-, and γ-catenin as well as p120. Infection of cells with various forms of PTPμ did not significantly alter the amounts of the catenins that co-immunoprecipitated with E-cadherin. As a control, a monoclonal antibody to chick L1 (8D9) was used. This antibody did not immunoprecipitate either E-cadherin or any of the catenins.

The PTPμ cytoplasmic domain interacts with RACK1

Using the yeast two-hybrid genetic screen, we identified an interaction between the cytoplasmic phosphatase domain 1 of PTPμ (PTPμD1) and RACK1 (receptors for activated protein C kinase) [54]. The interaction of PTPμD1 was mediated by protein-protein interactions and was not dependent upon phosphotyrosine. RACK1 is a homolog of the β subunit of heterotrimeric G proteins and is composed of WD-40 repeats [55]. Both Gβ and RACK1 form seven propeller structures (seven independently folding loops) proposed to mediate protein-protein interactions [56, 57]. RACK1 was originally identified as a protein that binds to activated protein kinase C (PKC) [55]. It has been suggested that activated PKC binding to RACK1 is required for the translocation of the enzyme to the plasma membrane, its physiologically relevant site of action [58].

More recent data suggests that RACK1 is a scaffolding protein that recruits a number of signaling molecules into a complex (Fig. 13). Theoretically, the seven propellers of the RACK structure could bind seven different proteins. RACK1 has been shown to bind PKC, PLCγ, *src* (a cytoplasmic PTK), cAMP phosphodiesterase-4, the β subunit of integrins, β chain of IL-5R (an interleukin receptor) and now PTPμ [54, 59-64]. RACK1 has also been demonstrated to bind select pleckstrin homology (PH) domains *in vitro* including dynamin, β spectrin and oxysterol binding protein (OSBP) [65]. PH domains are found in many signaling proteins and bind to the membrane in response to phosphatidyl-inositol-3-kinase (PI3K) activation. Some of the RACK1 interactions listed above have been shown to be mutually exclusive. This suggests that RACK1 may form distinct signaling complexes in response to unique cellular stimuli.

We characterized the association between RACK1 and PTPμD1 using the recombinant baculovirus expression system, and have shown that PTPμ binds to RACK1 in insect cells [54]. In addition, using cells which express endogenous PTPμ, we demonstrated that endogenous RACK1 and PTPμ associate [54]. PTPμ expression increases at high cell density in response to PTPμ-dependent adhesion in these cells. We observed an increased association of RACK1 with PTPμ as well as increased translocation of RACK1 to the plasma membrane at high cell density [54]. The interaction between PTPμ and RACK1 is not altered by PKC activation. Therefore, when RACK1 is bound to PTPμ it may still able to bind PKC. One could speculate that activation of PKC might be a downstream component of a PTPμ-induced cell adhesion-dependent signaling pathway.

The PTPμ cytoplasmic domain, regardless of catalytic activity, is required for the interaction with RACK1

Even though the presence of PTPμ does not alter the composition of the E-cadherin/catenin complex, it is possible that full length PTPμ regulates E-cadherin-dependent adhesion by recruiting other signaling molecules to the cadherin/catenin complex. In a recent paper [54], we demonstrated an interaction between the membrane-proximal phosphatase domain of PTPμ and RACK1 (Fig. 13), a receptor for activated PKC [55]. Since RACK1 binds to the catalytic domain of PTPμ, we tested whether catalytic activity of PTPμ was required to interact with RACK1. We performed immunoprecipitation with antibodies directed against PTPμ or RACK1 and subjected the immunoprecipitates to SDS-PAGE and immunoblotted the gels with anti-RACK1 antibodies. Immunoprecipitation of RACK1 showed that LNCaP cells infected with an empty vector expressed RACK1 (Fig. 14A, VEC), and that infection of cells with various forms of PTPμ did not alter the expression of RACK1 (Fig. 14A, WT, C-S and E, respectively). To investigate whether PTPμWT and the C-S mutant associate with RACK1 in LNCaP cells, we immunoprecipitated PTPμ using an antibody to the extracellular domain of PTPμ (BK2). RACK1 was found to associate with both PTPμWT (Fig. 14B, WT) and the C-S mutant (Fig. 14B, C-S), but not with PTPμ-extra (Fig. 14B, E). As expected, PTPμ antibody did not immunoprecipitate RACK1 from cells infected with an empty vector (Fig. 14B, VEC). This experiment was repeated with an antibody to the intracellular domain of PTPμ (SK7). As seen in Figure 14C, the SK7 antibody also co-immunoprecipitated RACK1 from cells infected with PTPμWT or C-S, but not from cells infected with PTPμ-extra or an empty vector. Taken together, these data demonstrate that full length PTPμ regardless of its catalytic activity associates with RACK1.

The association between PTPμ and RACK1 suggests that the presence of the PTPμ protein in LNCaP cells may regulate the PKC pathway, which could be involved in the restoration of E-cadherin-mediated adhesion by PTPμ. Several studies have shown that activation of the PKC pathway can either upregulate or downregulate E-cadherin-mediated adhesion depending on cell type [66]. Therefore, we investigated whether activation of PKC by PMA affects the ability of PTPμ to restore E-cadherin-mediated adhesion. LNCaP cells infected with retrovirus containing PTPμWT were allowed to adhere to PTPμ, Laminin or E-cadherin as described above. The cells were then treated with PMA for 15 min. to activate PKC, which did not affect adhesion to either PTPμ or Laminin (Fig. 15). However, activation of PKC detached the PTPμ-expressing cells from E-cadherin (Fig.15). The statistical analyses for the effects of PMA on LNCaP adhesion can be found in [50].

Inhibition of PKCδ induced LNCaP adhesion to E-cadherin

To further examine the signal transduction pathways involved in restoring E-cadherin-mediated adhesion, we studied the effect of various kinase inhibitors on the ability of LNCaP cells to adhere to E-cadherin. Uninfected LNCaP cells were added to coverslips with immobilized E-cadherin, and the cells were subsequently treated with the inhibitors. Chelerythrine chloride and GF109203X are broad specificity compounds that inhibit most PKC isoforms. Rottlerin only inhibits the PKCδ isoform of PKCs. We found that treatment of the cells with three different PKC inhibitors, chelerythrine chloride, GF109203X, and a PKCδ-specific inhibitor (Rottlerin) induced LNCaP adhesion to E-cadherin (Fig. 16B, CHE, GF and R, respectively). The induction of E-cadherin-dependent adhesion was due to inhibition of PKCs in general but we believe specific for inhibition of PKCδ, since treatment of LNCaP cells with the PKCα- and PKCβ-specific inhibitor Gö6976 did not induce adhesion (Fig. 16B, Go). In addition, neither DMSO nor the PI 3-kinase inhibitor LY294002 induced LNCaP adhesion to E-cadherin (Fig. 16B, DMSO and LY, respectively). Also, the effect of the PKCδ inhibition was specific in that it only affected the ability of LNCaP cells to adhere to E-cadherin, but did not alter adhesion to Laminin (data not shown). Interestingly, the PMA-induced detachment of the PTPμ-expressing cells from E-cadherin was blocked by pre-

incubating the cells with Rottlerin (data not shown). Furthermore, PKCδ was found to associate with RACK1 in LNCaP cells, although this association was not affected by the presence of either PTPμWT or the C-S mutant (Fig. 14D) [55]. This result is not surprising since our experiments were done in the presence of serum, which activates PKC. Our data suggests that PTPμ negatively regulates PKCδ activity to restore E-cadherin-dependent adhesion. However, the precise mechanism of PKCδ regulation by PTPμ is not clear but is likely to involve RACK1. Taken together, these data indicate that PTPμ may restore E-cadherin-mediated adhesion in LNCaP cells by regulating the PKC pathway through the recruitment of RACK1 to the PTPμ complex.

Determination of the function of PTPμ in the N-cadherin complex

Our previous studies have shown that PTPμ associates with N-cadherin and regulates N-cadherin-mediated neurite outgrowth. Therefore, it is possible that loss of PTPμ expression may result in defects in N-cadherin-mediated adhesion. LNCaP cells express N-cadherin (Fig. 3). However, these cells are defective in N-cadherin-dependent adhesion (Fig. 4). We found that PTPμ re-expression also induced adhesion to N-cadherin (Fig. 17F). Similar to our results with E-cadherin, induction of adhesion to N-cadherin did not require phosphatase activity (Fig. 17J). These results are shown graphically in Figure 18. The most surprising finding is that PTPμ-extra, which did not induce adhesion to E-cadherin, did restore adhesion to N-cadherin (Fig. 17M).

Our preliminary results suggest that inhibition of PKCα, β (based upon Gö6976) and PKC δ (based upon Rottlerin) do not induce adhesion to N-cadherin (Fig. 19). Since the PKC inhibitor GF109203X did induce adhesion to N-cadherin that suggests that a PKC isoform is involved (Fig. 19). Based upon the profile of PKC isoforms that are inhibited by GF109203X (see Calbiochem catalog), these data suggest that PKCε may be the isoform that induces adhesion to N-cadherin. The inhibitors were used at the following concentrations: 5μM Rottlerin, 10μM chelerythrine chloride, 0.5μM GF109203X, 15 nM Gö6976 or DMSO alone. At the concentrations used, chelerythrine chloride and GF109203X are specific for PKC, whereas Rottlerin is specific for PKCδ and Gö6976 is specific for PKCα and ß.

Tyrosine phosphorylation by the *src* PTK negatively regulates cadherin-dependent adhesion [6, 7, 9], however, the mechanism is unknown. Interestingly, RACK1 binds to the *src* PTK. We recently found that *src* and PTPμ compete for binding to RACK1 [54]. Since *src* is known to negatively regulate cadherin-dependent adhesion, the ability of PTPμ and *src* to compete for RACK1 may indirectly affect tyrosine phosphorylation of the cadherin complex. This may be an important point since even the catalytically inactive forms of PTPμ restored E-cadherin-dependent adhesion (Fig. 11). The catalytically inactive form of PTPμ still binds to RACK1 (Fig. 14). Therefore, an active phosphatase may not be required to restore adhesion because displacement of the *src* PTK may be sufficient to alter the tyrosine phosphorylation of the cadherin complex.

RACK1 binds to the SH2 domain of *src* via a phosphotyrosine (Tyr 246) in the sixth WD repeat of RACK1 [63, 64]. Tyrosine phosphorylation of RACK1 is enhanced by both growth factors and phorbol ester stimulation of PKCs. Interestingly, PKCδ is tyrosine phosphorylated by src, which results in changes in membrane association and its downregulation. The presence of the PTPμ protein could recruit RACK1 to the plasma membrane where it could dissociate from PTPμ and possibly bind to and inactivate src. Inactivation of *src* could indirectly regulate the tyrosine phosphorylation of either the cadherins, catenins or PKCs thereby restoring cadherin-mediated adhesion. We have not observed direct changes in tyrosine phosphorylation by immunoprecipitation/immunoblotting. However, we did use the *src* tyrosine kinase inhibitor PP1 to treat LNCaP cells and measure cell adhesion. We found that PP1 treatment of LNCaP cells restored adhesion to both E-cadherin and N-cadherin (Fig. 19) indicating a role for the *src* tyrosine kinase in regulating cadherin-dependent adhesion in prostate cells.

II. Elucidate the signal transduction pathway that PTPμ utilizes to negatively regulate cell growth

The mechanisms underlying growth inhibition are not understood, but it possible that it may occur through inhibition of mitogenic signals initiated from growth factor receptors [67]. Growth factor stimulation of cell growth involves binding of ligand to the growth factor receptor followed by receptor dimerization, autophosphorylation and trans-phosphorylation (see Fig.20). These ligand induced phosphorylation events recruit adaptor proteins such as SH2 domains and PTB domains that bind phosphotyrosine residues. The recruitment of adaptor proteins such as Shc, Grb2 and SOS is involved in activation of the Ras/MAPK pathway. RPTK stimulation also recruits the phosphotyrosine binding proteins PLCγ, which activates PKC, and the p85 (regulatory) subunit of PI3K. The PI3K enzyme has regulatory (p85) and catalytic (p110) subunits. RACK1 has already been shown to bind PLCγ. It was important to determine if PLCγ or PI3K pathways were regulated by re-expression of PTPμ in LNCaP cells. I have already discussed how we studied the PKC pathway in aim I. Investigating the PI3K pathway is important because of our results in which PI3K inhibitors block PTPμ-dependent adhesion. In addition, the interaction of RACK1 with PH domains suggests that PI3K signaling will be an important way to regulate the PH domain-containing proteins in the RACK1 signaling complex. Therefore, we investigated a role for PTPμ in regulating the PI3K pathway.

A. Re-express wild type PTPμ in LNCaP cells and determine the expression and/or the activation state of signaling proteins in the PI3K pathway.

PI3K activation results in the accumulation of PIP-3 at the plasma membrane. Interestingly PTEN, originally identified as a tyrosine phosphatase, is a tumor suppressor gene in many types of cancer including prostate [68]. PTEN was later shown to be a lipid phosphatase that dephosphorylates PIP-3 [69]. LNCaP cells are phenotypically null for PTEN and may have activated PI3K signaling [70]. Clearly, regulation of the PI3K pathway is important in normal prostate cells. PIP-3 formation recruits proteins with PH domains to the membrane [69]. It is interesting to note that RACK1 binds to select plekstrin homology (PH) domains [65]. PH domains are found in a number of signaling proteins that regulate cell growth including AKT [71]. The serine/threonine kinase, AKT, is downstream of PI3K activation and acts as a survival factor or anti-apoptotic factor [72]. Interestingly, PTPμ has a consensus site for AKT phosphorylation in the first phosphatase domain (Thr^{939}). It is possible that AKT and PTPμ may regulate enzymatic activity of one another. Since PTPμ negatively regulates cell growth and the PI3K pathway is known to be activated in LNCaP cells [70], overexpression of wild type PTPμ may overcome the PI3K activation to negatively regulate growth. The regulation of the PI3K pathway by PTPμ may be mediated via RACK1-induced association with AKT.

1. Analyze cell cycle changes when PTPμ is re-expressed in LNCaP cells

To elucidate the molecular mechanism of PTPμ-dependent growth regulation, we needed to analyze which stage of the cell cycle is affected. We analyzed cell cycle progression by flow cytometry using LNCaP cells infected with vector or wild type PTPμ since we only see negative growth regulation by wild type PTPμ. We infected LNCaP cells with wild type PTPμ in the presence or absence of tetracycline, which controls expression of the PTPμ gene, to verify that growth inhibition is dependent upon PTPμ expression. In addition, we analyzed the growth of LNCaP cells infected with wild type PTPμ in the presence or absence of PI3K inhibitors.

2. Examine the activation state of proteins in the PI3K pathway

One of the hallmarks of PI3K signaling is activation of AKT. Activated AKT downregulates the cyclin/cyclin-dependent kinase (Cdk) inhibitor p27 and inactivates Gsk-3 (a serine/threonine kinase) which stabilizes cyclin D1 and leads to increased proliferation [73]. PTEN can induce G1 arrest by upregulation of p27, which associates with cyclin E/Cdk2 complexes and inhibits Rb (retinoblastoma gene product) phosphorylation [73]. If PTPμ is regulating the PI3K pathway via

inactivation of AKT, we should detect changes in p27, cyclin E and cyclin D1 protein levels. In addition since other proteins may be affected in response to PTPμ re-expression, we looked at protein expression of all the cyclins and Cdk inhibitors.

Methods: Re-expression of wild type PTPμ may be negatively regulating cell growth by inducing changes in the activation state of some signaling protein in the PI3K pathway. The analysis of cell cycle regulatory proteins was compared between LNCaP cells expressing wild type and catalytically inactive PTPμ. We utilized phospho-specific antibodies on protein lysates to examine expression and activation of AKT (UBI, Cell Signaling and Biosource). The cyclin proteins and Cdk inhibitors are either regulated by protein expression or degradation. Therefore, we immunoblotted cell lysates to determine if there were changes in expression of cyclins and Cdk inhibitors using antibodies from Transduction Labs or UBI. Specifically, we focused on determining if there were changes in cyclin D1 and cyclin E expression. In addition, we examined the expression of the Cdk inhibitors, p27 and p21 in LNCaP cells following re-expression of PTPμ.

B. Utilize PI3K inhibitors to determine if the PI3K pathway is regulated by wild type PTPμ.

Methods: We treated PTPμ-expressing LNCaP cells with the PI3K inhibitors wortmannin (Sigma) and LY294002 (LC laboratories) to determine if they block the ability of PTPμ to negatively regulate cell growth. Wortmannin is used at 50nM and incubated for 30 min. at 37°C. LY294002 is used at 10μM and incubated for 1hr. at 37°C. To support the idea that PI3K signaling is activated in LNCaP cells, we infected cells with a dominant negative p85 subunit of PI3K (missing the region that binds the catalytic subunit) and tested the affect it has on LNCaP cells. We generated a dominant negative p85 construct using the same retroviral system described above. The dominant negative p85 retrovirus was used to infect LNCaP cells and determine if it negatively regulates cell growth by blocking the PI3K signaling pathway.

Results from Aim II:

The PTPμ phosphatase negatively regulates growth

We generated retroviruses encoding these PTPμ mutants tagged at the C-terminus with the green fluorescence protein (GFP) (Fig. 5). All of the forms of PTPμ were expressed (Fig. 6) and properly localized at the cell surface (Fig. 7). The PTPs share a catalytic domain with ~30% amino acid identity that is characterized by the sequence motif {(I/V)HCXAGXXR(S/T)G}which forms the phosphate binding pocket. The cysteine residue sits at the base of the active site cleft and is essential for catalysis. Mutations can be made that alter either the affinity for substrate (K_m) or the rate of catalysis (V_{max}). The "substrate trapping" mutant (D-A) retains normal affinity for its substrate but catalytic activity is reduced resulting in irreversible binding of the substrate. Mutations of the conserved cysteine residue (C-S) result in a catalytically active form of the phosphatase (Fig. 5). Mutation of a conserved arginine residue (R-M) acts as a "dominant negative". The mutant forms of PTPμ are useful for determining its physiological substrates and function.

Growth rates of cells infected with wild type or mutant forms of PTPμ were analyzed. Re-expression of wild type PTPμ negatively regulated the growth of LNCaP cells (Fig.21). However, the mutant forms of PTPμ with altered catalytic activity did not affect cell growth. These data suggest that PTPμ phosphatase activity is required to negatively regulate cell growth. Wild type PTPμ appeared to induce a saturation density effect on the cells. A reduction in growth rate when cells reach high density or contact one another defines contact inhibition of cell growth. Our data suggested that re-expression of PTPμ in LNCaP cells did not induce apoptosis. If PTPμ is inhibiting growth, it may be affecting the G1 to Go transition or the G1 to S phase transition. Our

data suggested that PTPμ affects the G1 to Go transition of the cell cycle. This is intriguing because the mutants have allowed us to separate the effect of PTPμ on cell growth (phosphatase-dependent) and cadherin-dependent cell adhesion (requires the intracellular domain).

Our results suggest that PI3K signaling is likely to regulate a PTPμ-dependent process. We recently found that inhibition of PI3K blocks adhesion to PTPμ (Fig. 22). This suggests that PI3K may regulate a PTPμ-dependent signal required to mediate adhesion. It is unclear whether PI3K is upstream or downstream of PTPμ. However, since one PTPμ-dependent process is regulated by PI3K, it is possible that the negative growth regulation mediated by PTPμ may involve the PI3K pathway. We tested whether PI3K is downstream of a PTPμ signal by testing the effects of PI3K inhibitors on the ability of PTPμ to negatively regulate growth. We have been unable to detect changes in the downstream PI3K effectors such as AKT in response to PTPμ addition. It is likely that these changes are very transient and difficult to measure by re-expression of PTPμ. We did not detect any changes in growth in response to PI3K inhibition. We are pursuing other pathways that PTPμ may utilize to negatively regulate cell growth.

KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstrating that PTPμ expression is altered in a subset of prostate cancer cell lines
2. Determining that E-cadherin and N-cadherin-dependent adhesion is nonfunctional in LNCaP cells
3. Restoring PTPμ expression to LNCaP cells and testing its effect on cell adhesion
4. Demonstrating that PTPμ re-expression induces calcium-dependent aggregation
5. Determining that PTPμ is required for both E- and N-cadherin-dependent adhesion
6. Demonstrating that PTPμ's cytoplasmic domain is required for E-cadherin-dependent adhesion
7. Determining that PTPμ restores E-cadherin dependent adhesion by recruiting RACK1 to the complex
8. Demonstrating that PKC delta inhibition restores adhesion to E-cadherin
9. Determining that PTPμ restores adhesion to N-cadherin by a different mechanism involving its juxtamembrane domain, src and PKC epsilon.
10. Demonstrating that wild type PTPμ negatively regulates growth
11. Determining that PI3K regulates PTPμ-dependent adhesion

REPORTABLE OUTCOMES:

Mourton, T., Hellberg, C., Burden-Gulley, S., Hinman, J., Rhee, A. and Brady-Kalnay, S. The protein tyrosine phosphatase, PTPμ, binds and recruits RACK1 to points of cell-cell contact. *Journal of Biological Chemistry,* 276, 14896-14901. 2001.

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CONCLUSIONS:

The experiments described in this report identified target pathways utilized by PTPμ to restore adhesion and negatively regulate cell growth. In addition, the experiments should serve to focus future mechanistic studies on the relevant target signaling proteins downstream of PTPμ. Identifying downstream components of a PTPμ signal will identify additional proteins whose expression may be regulated during progression of prostate cancer that could be used as disease

stage markers. These studies identified potential therapeutic targets for treatment of prostate cancer that may mimick PTPμ re-expression in LNCaP cells.

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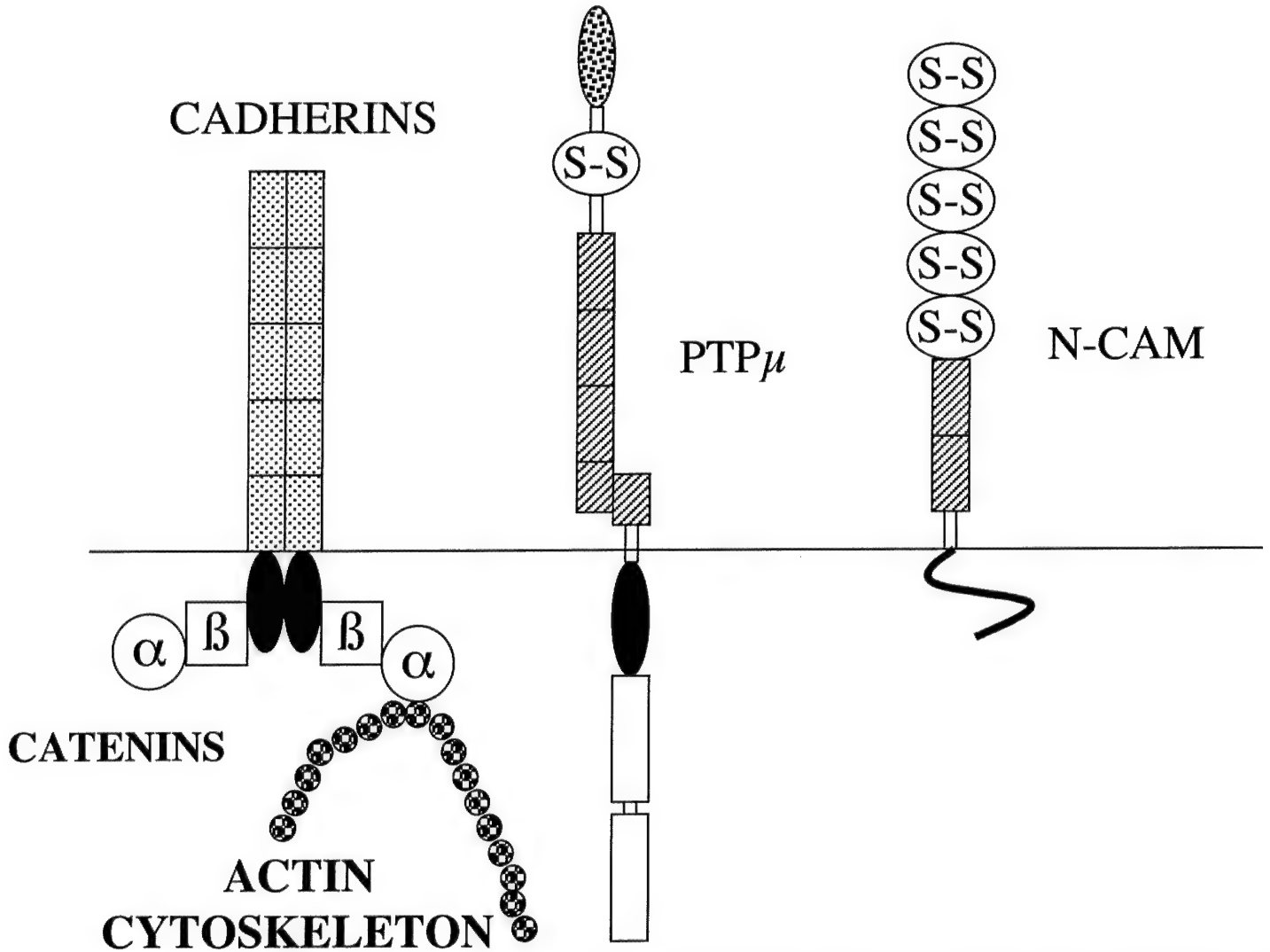


Fig.1. Schematic diagram of PTPμ, N-CAM and the cadherin/catenin complex. Cadherins exist as dimers and bind to β catenin and via α catenin to actin. The S-S circle represents the Ig domain. The diagonal hatched rectangle is an FNIII repeat. The open rectangles are PTP domains. The black oval is the cadherin-like intracellular domain. The stippled oval in PTPμ is the MAM domain. The cadherin extracelllular domain contains 5 EC repeats (stippled rectangles).

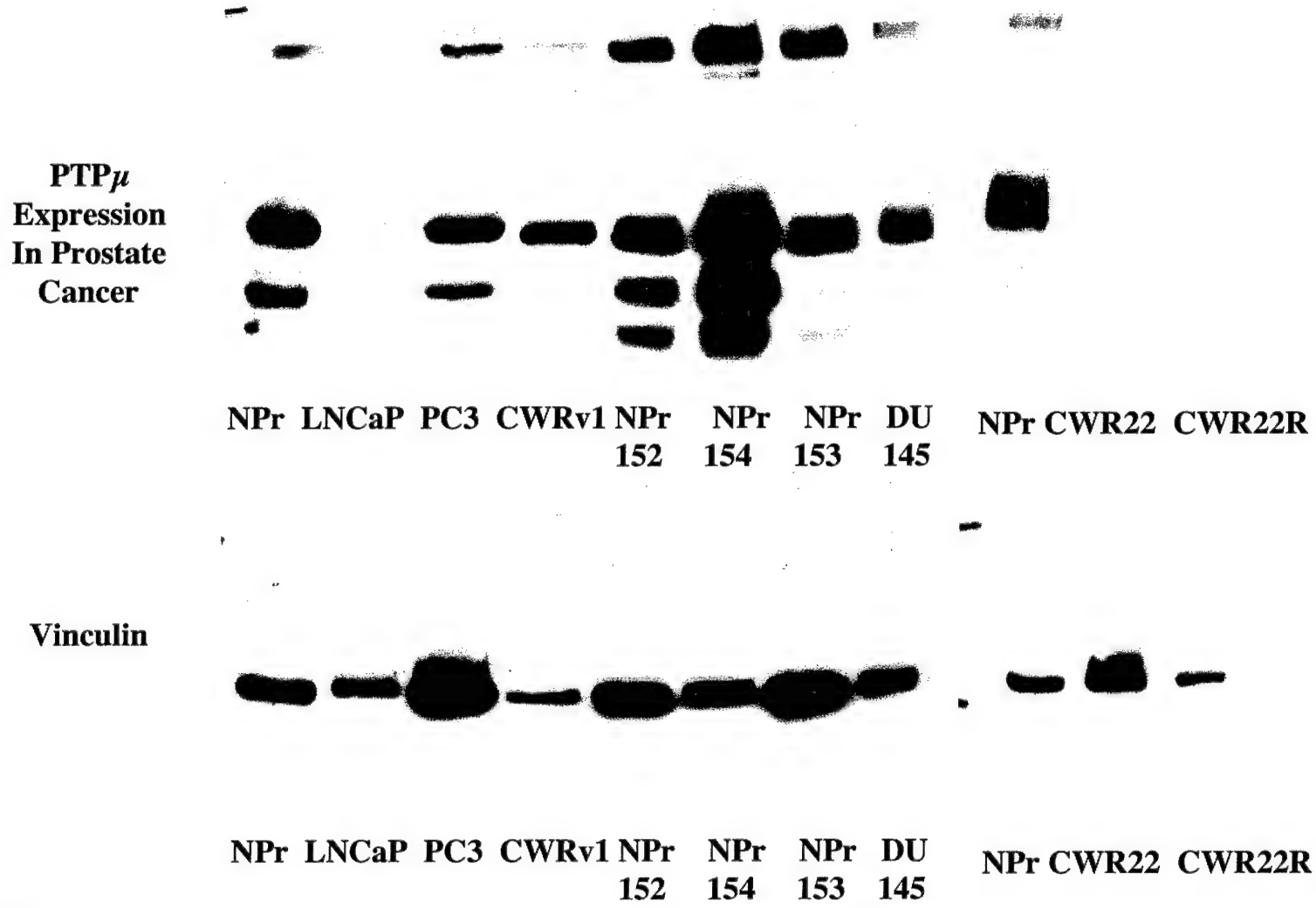


Fig.2. Prostate cancer cell lines are immunoblotted for PTPμ or vinculin (control). Changes in PTPμ expression were observed in LNCaP cells and two xenografts (CWR22 & CWR22R).

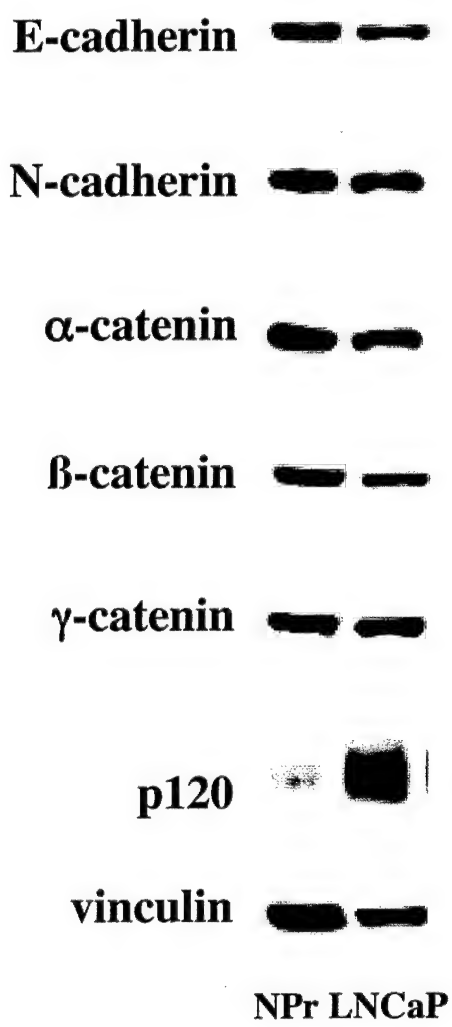


Fig. 3. Expression of cadherins and catenins. Normal prostate (NPr) and parental LNCaP cells were compared by immunoblotting with the respective antibodies.

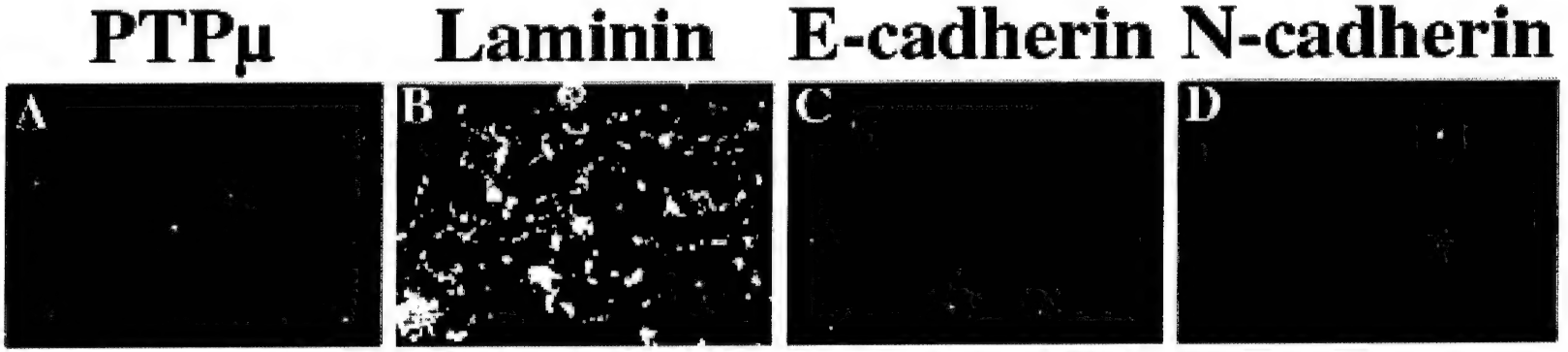


Fig. 4. Adhesion of Parental LNCaP cells to purified proteins. LNCaP cells adhere to Laminin but not PTPμ, E-cadherin or N-cadherin.

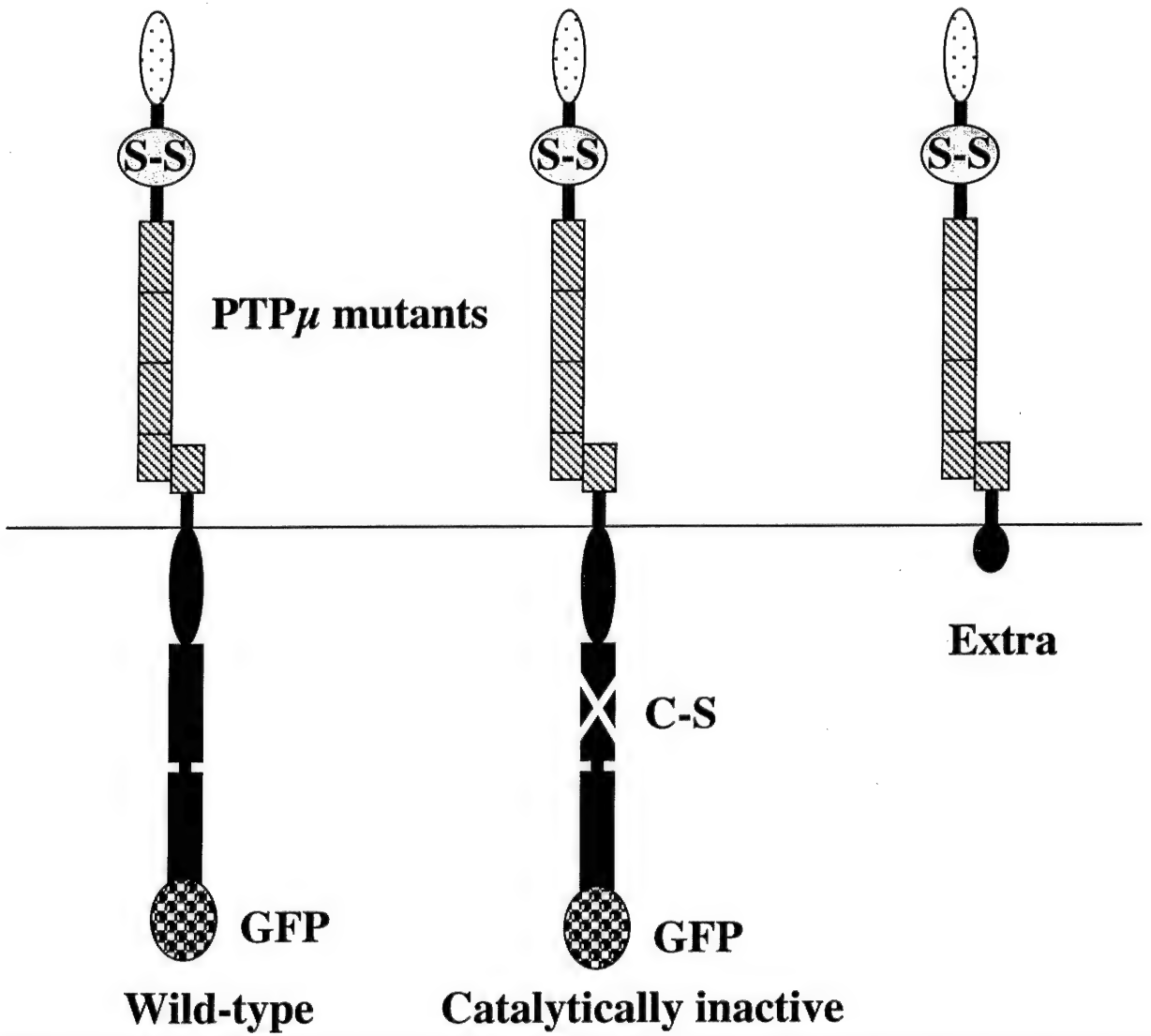


Fig. 5. These PTPμ mutants were constructed using a retroviral vector system. Shown diagrammatically are the wild type PTPμ (WT), the C-S and the Extra PTPμ mutants. The wild type and C-S mutant are tagged with the green fluorescent protein (GFP) at their C-terminus.

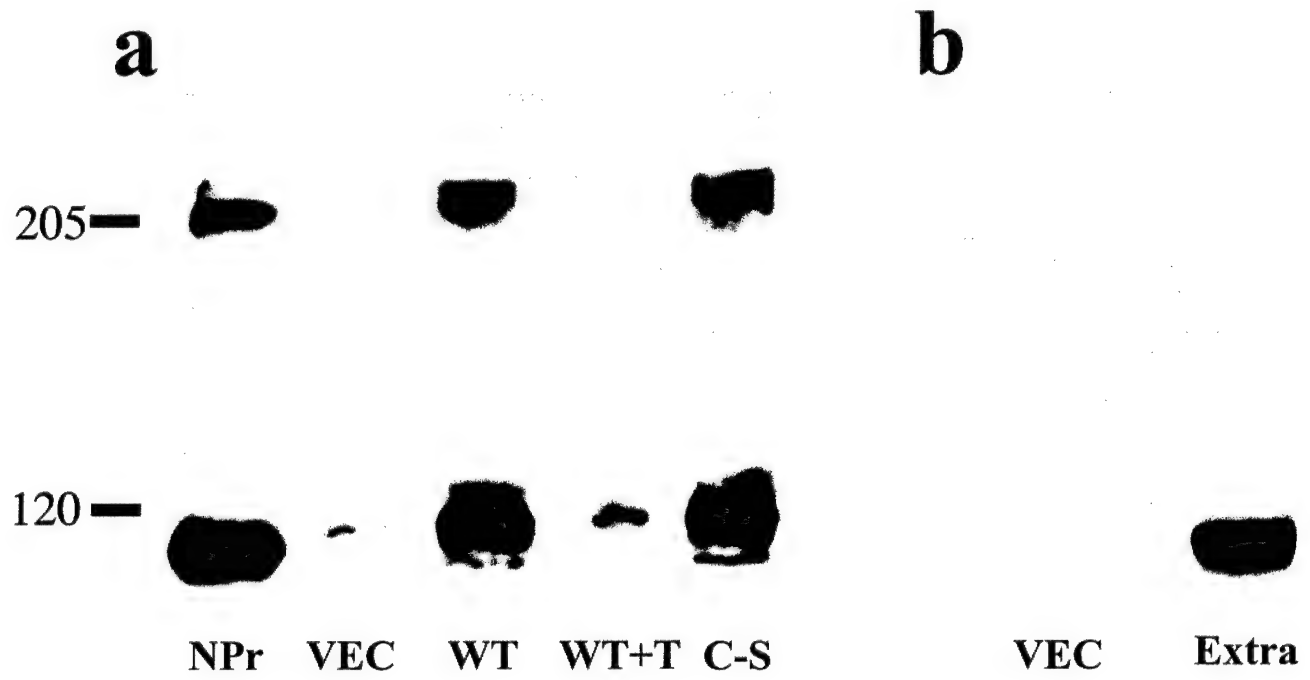


Fig.6. Re-expression of the wild type and mutant forms of PTPμ in LNCaP cells. Panels A and B are immunoblots using antibodies to the intracellular domain of PTPμ (a) and the extracellular domain of PTPμ (b). The cells were grown either in the absence or in the presence (+T) of tetracycline.

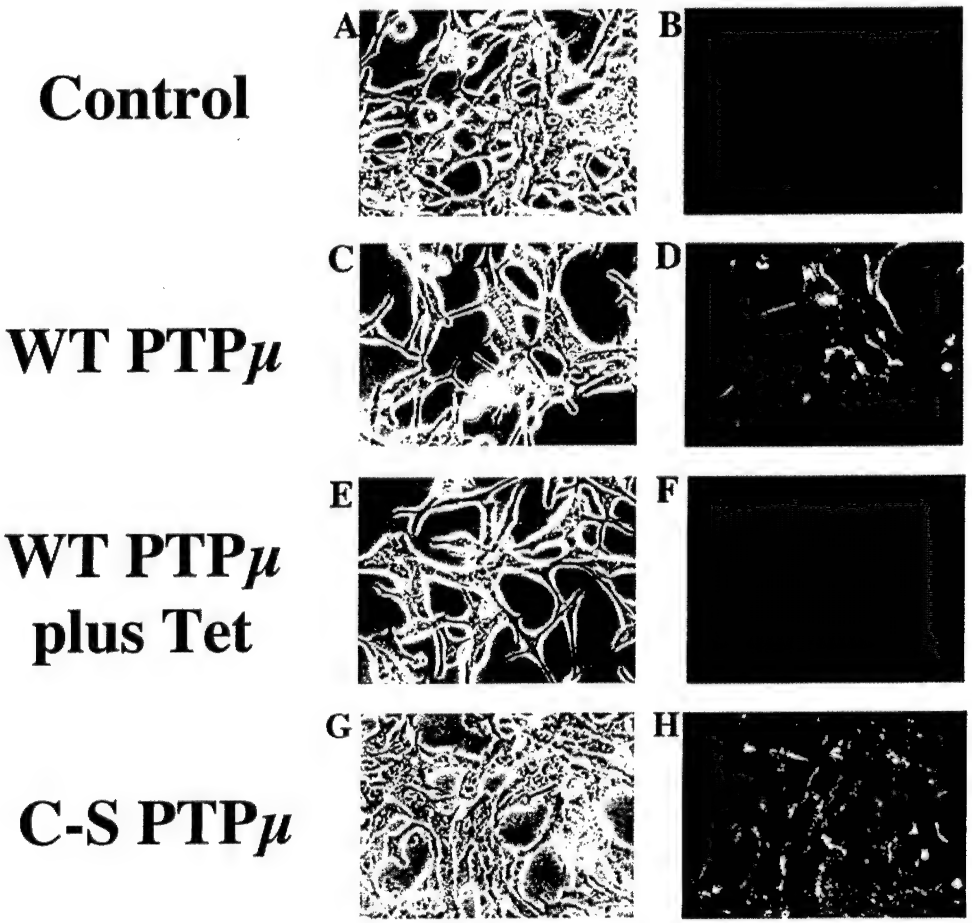


Fig.7. Re-expression of PTPμ in LNCaP cells as examined by fluorescence microscopy. A and B are vector-infected cells. C-F are wild type PTPμ infected cells. In panels E and F, the cells are grown in the presence of tetracycline. Panels G and H are C-S PTPμ infected cells.

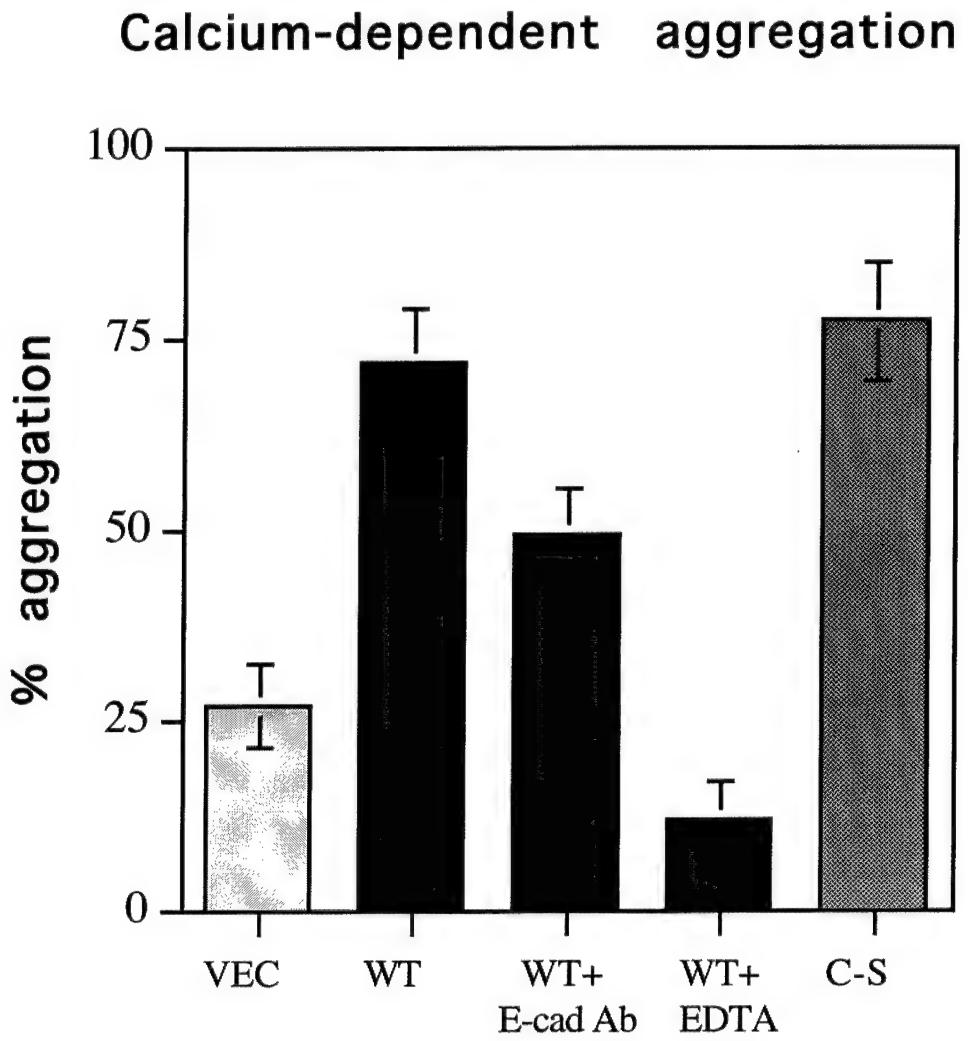


Fig.8. Re-expression of PTPμ in LNCaP cells induced calcium-dependent aggregation in LNCaP cells. The aggregation could be moderately inhibited by antibodies to E-cadherin or aggregating the cells in the presence of EDTA.

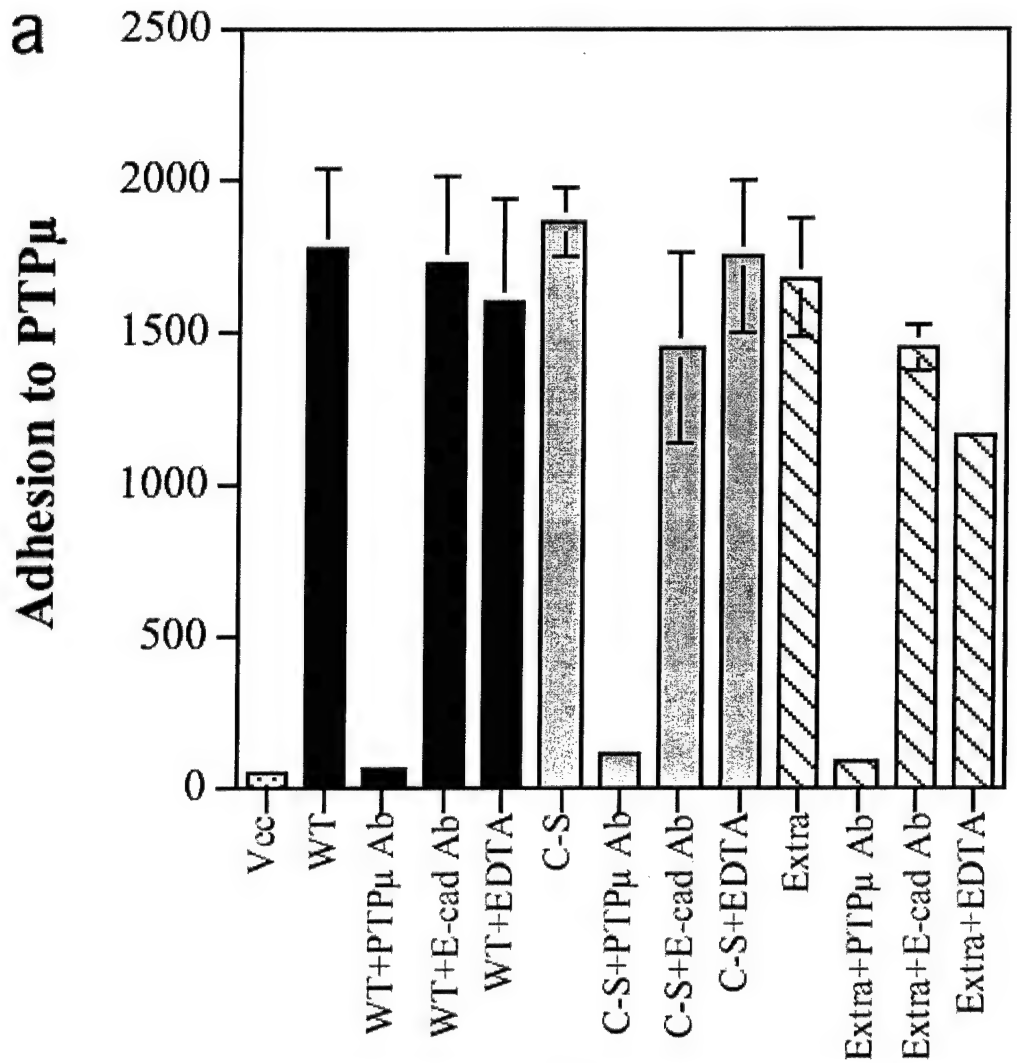


Fig.9. Re-expression of all three forms of PTPμ in LNCaP cells induced adhesion to PTPμ. The adhesion could be inhibited by antibodies to PTPμ but the presence of EDTA had no effect on adhesion to PTPμ as expected.

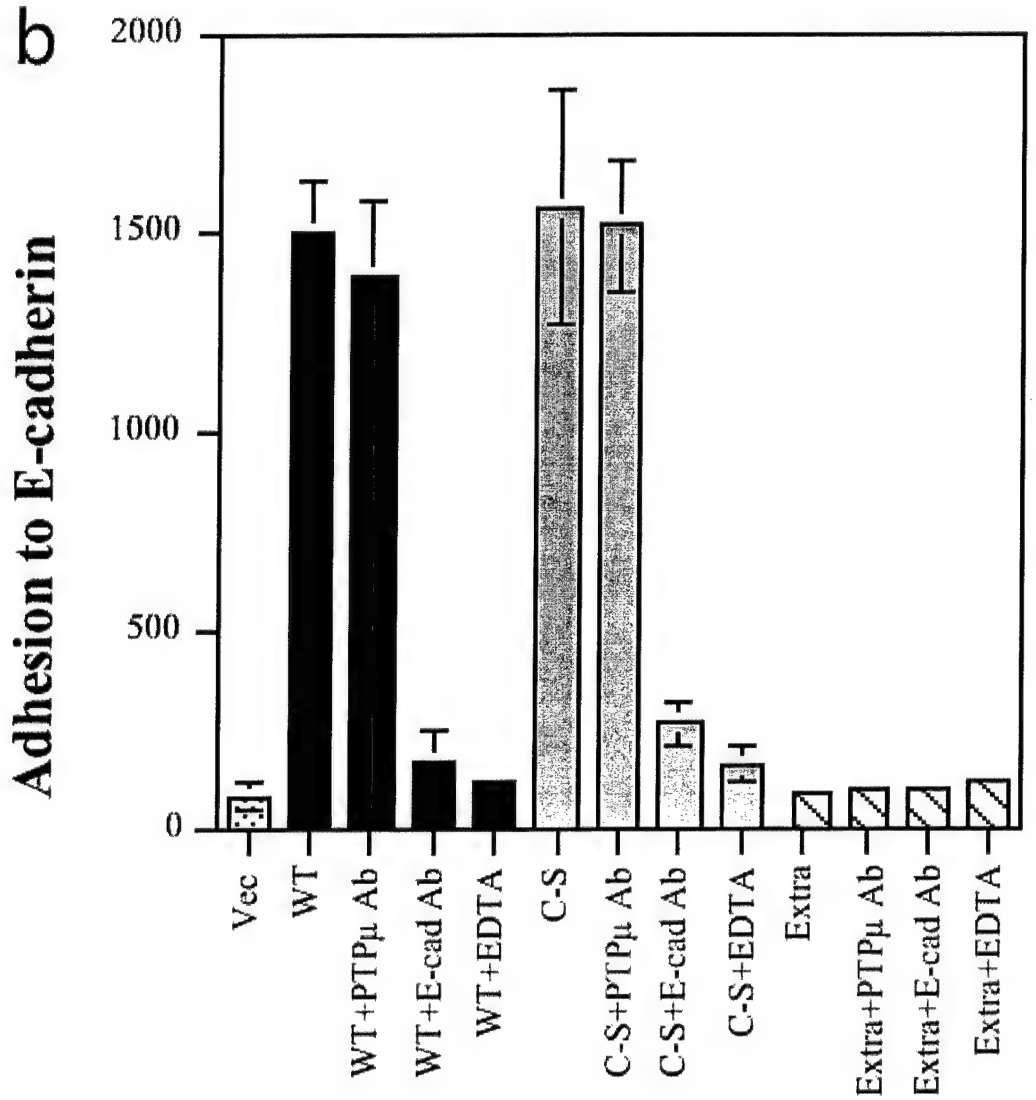


Fig.10. Re-expression of wild type and C-S PTPμ in LNCaP cells induced adhesion to E-cadherin. The adhesion could be inhibited by antibodies to E-cadherin or EDTA.

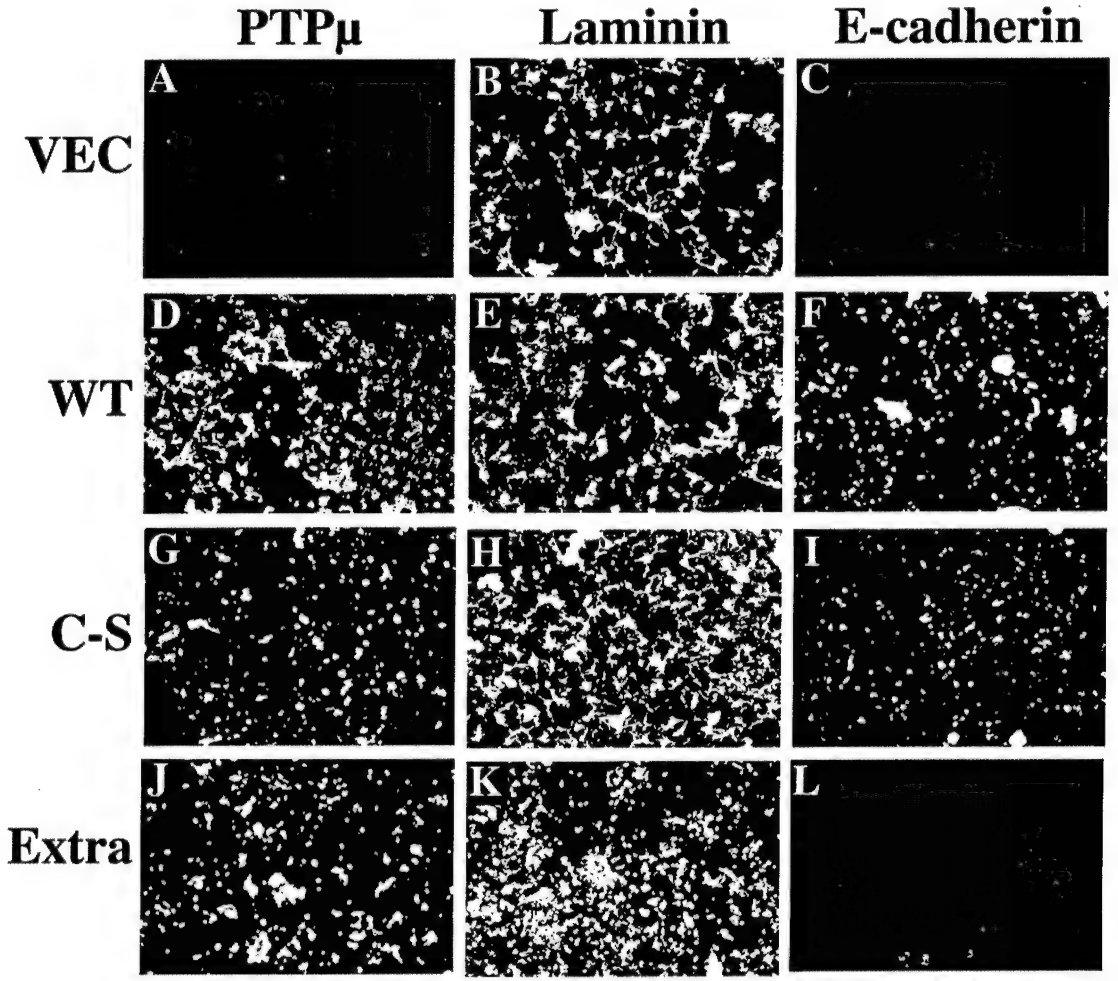


Fig.11. Re-expression of wild type and C-S PTPμ in LNCaP cells induced adhesion to E-cadherin. Extra PTPμ did not induce adhesion to E-cadherin however it did induce adhesion to PTPμ as expected.

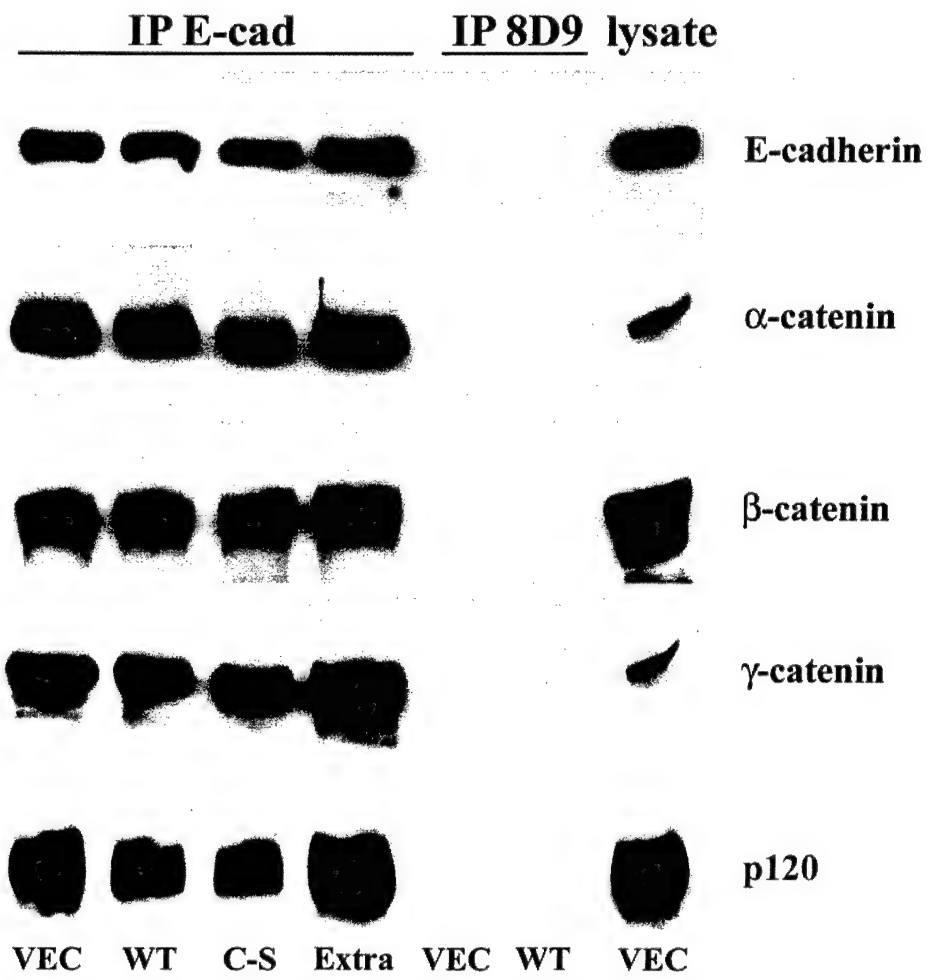


Fig. 12. Expression of cadherins and catenins was not altered by re-expressing PTPμ. In addition, the cadherin-catenin complex was unaltered by re-expression of PTPμ as assessed by immunoprecipitation using E-cadherin antibodies followed by immunoblotting with cadherin or catenin antibodies.

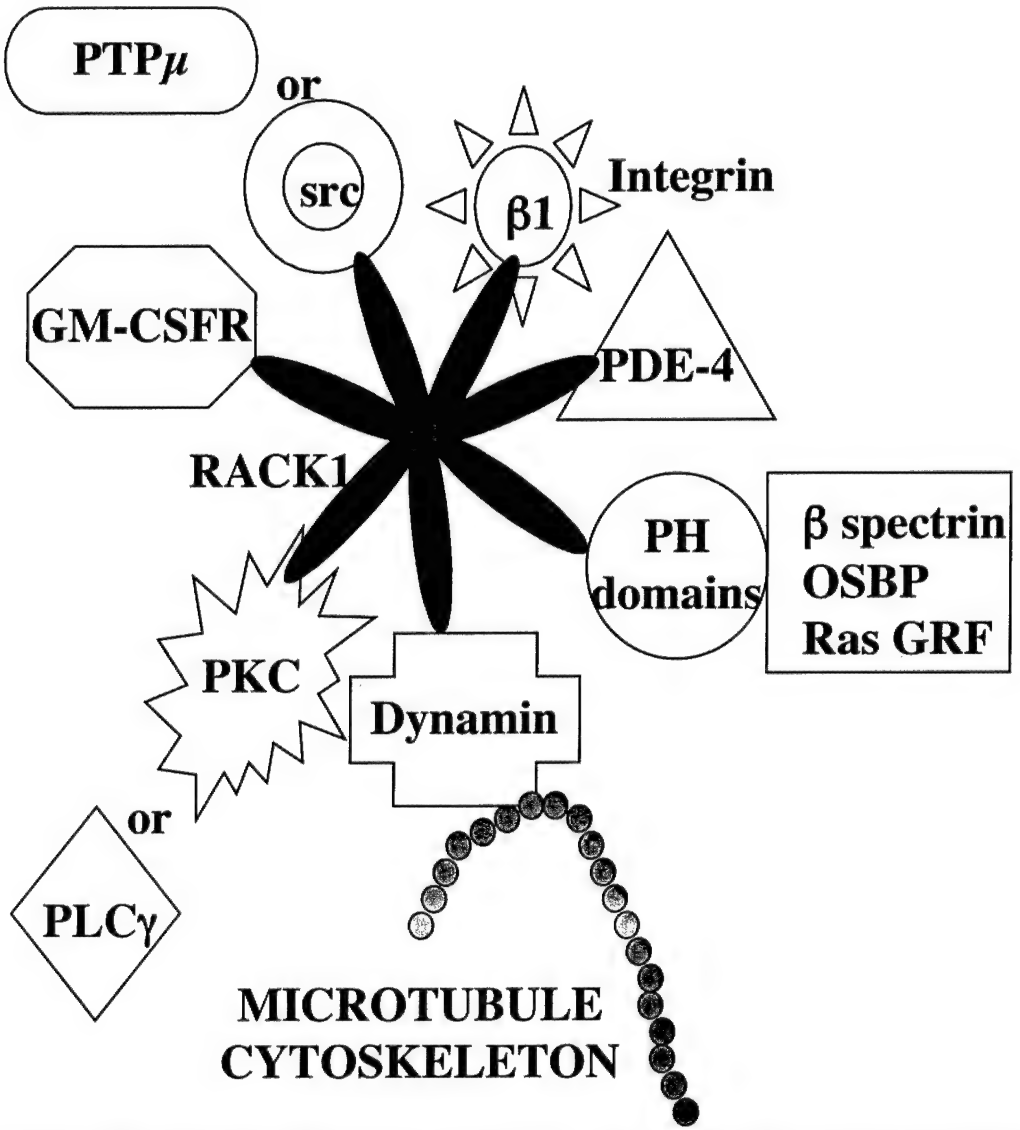


Fig. 13. RACK1 and its associated proteins. RACK1 is shown in black with seven propellers. RACK1 binds PKC or PLCγ; PTPμ or *src*; PDE-4; select PH domains including β spectrin; the IL-5R; and the β subunit of integrins. Mutually exclusive interactions exist between PTPμ and src as well as between PLCγ and PKC.

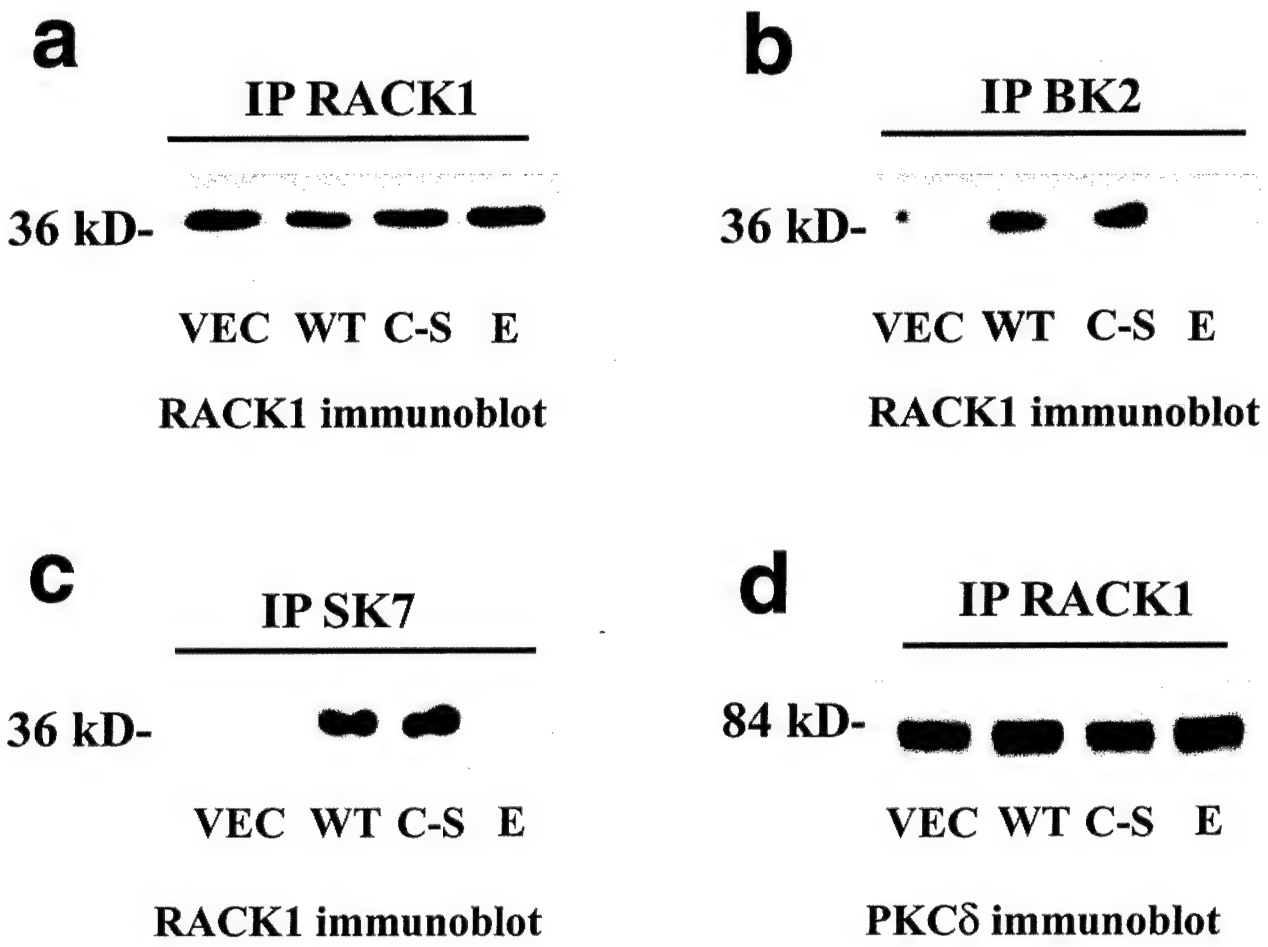


Fig. 14. RACK1 associates with PTPμ only in LNCaP cells expressing wild type and C-S but not Extra. Immunoprecipitations (IP) with various antibodies are immunoblotted with the antibodies indicated below each panel. PKCδ and RACK1 interact under all conditions.

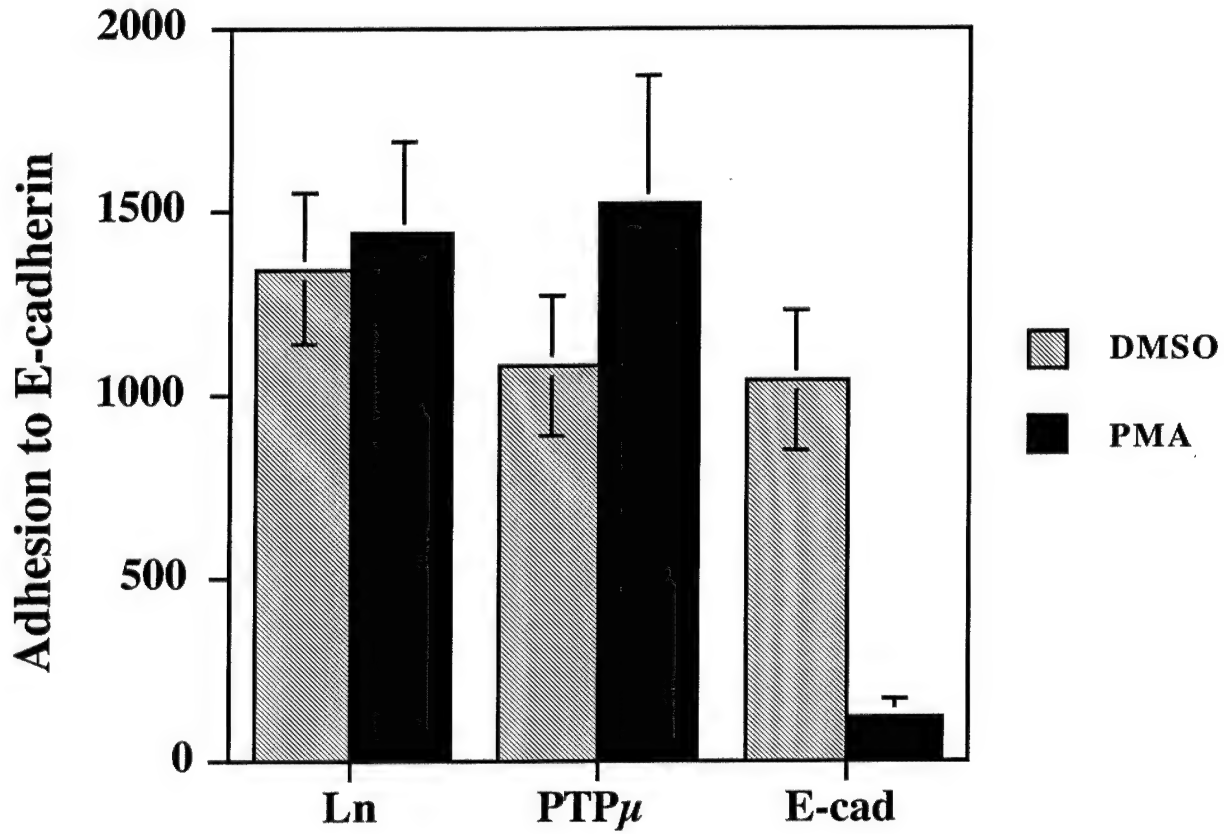
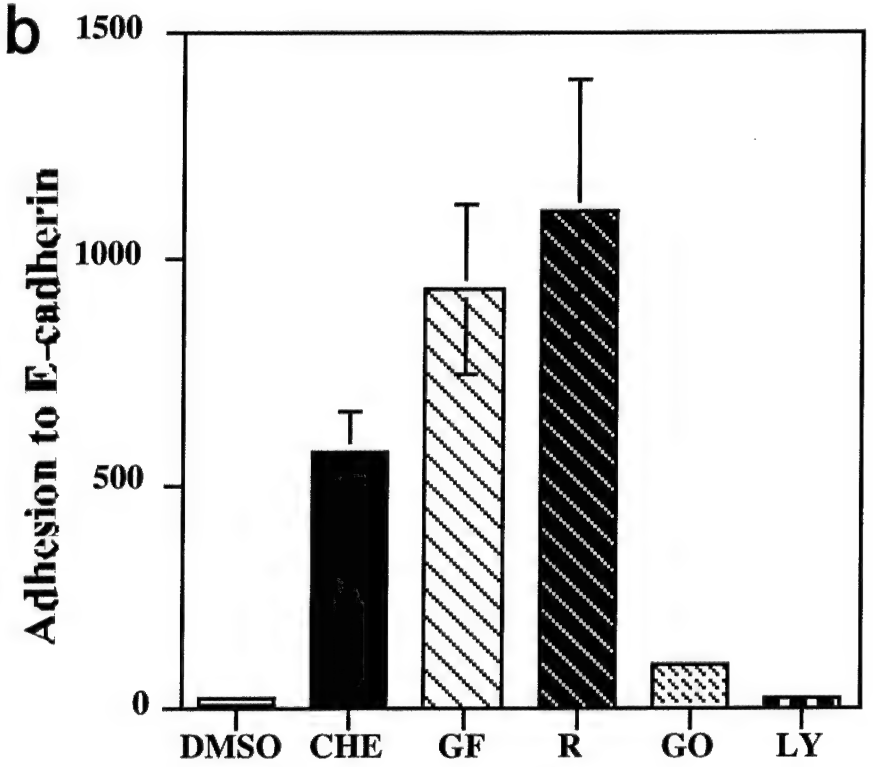


Fig. 15. Adhesion to various purified proteins when cells expressing PTPμ wild type are treated with control (DMSO) or PMA to activate protein kinase C. Only adhesion to E-cadherin is affected by PKC stimulation. PKC activation inhibits adhesion to E-cadherin.



Inhibition of PKCδ induces adhesion to E-cadherin in uninfected LNCaP cells

Fig. 16. Adhesion to E-cadherin is induced in parental LNCaP cells by inhibition of protein kinase C. Generic PKC inhibitors (CHE and GF) and the PKCδ specific inhibitor, Rottlerrin (R), induce adhesion to E-cadherin.

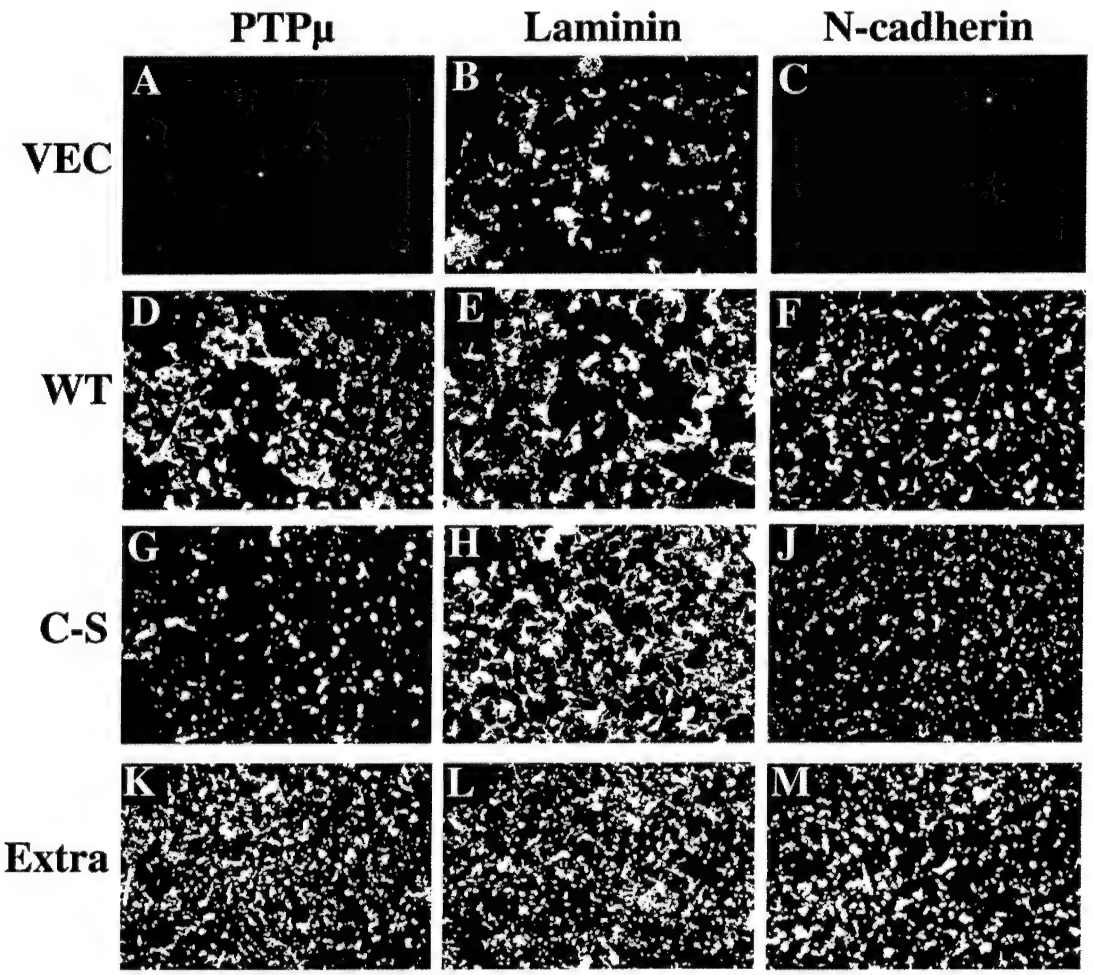


Fig.17. Re-expression of wild type, C-S and Extra PTPμ in LNCaP cells induced adhesion to N-cadherin. All forms of PTPμ induced adhesion to PTPμ as expected.

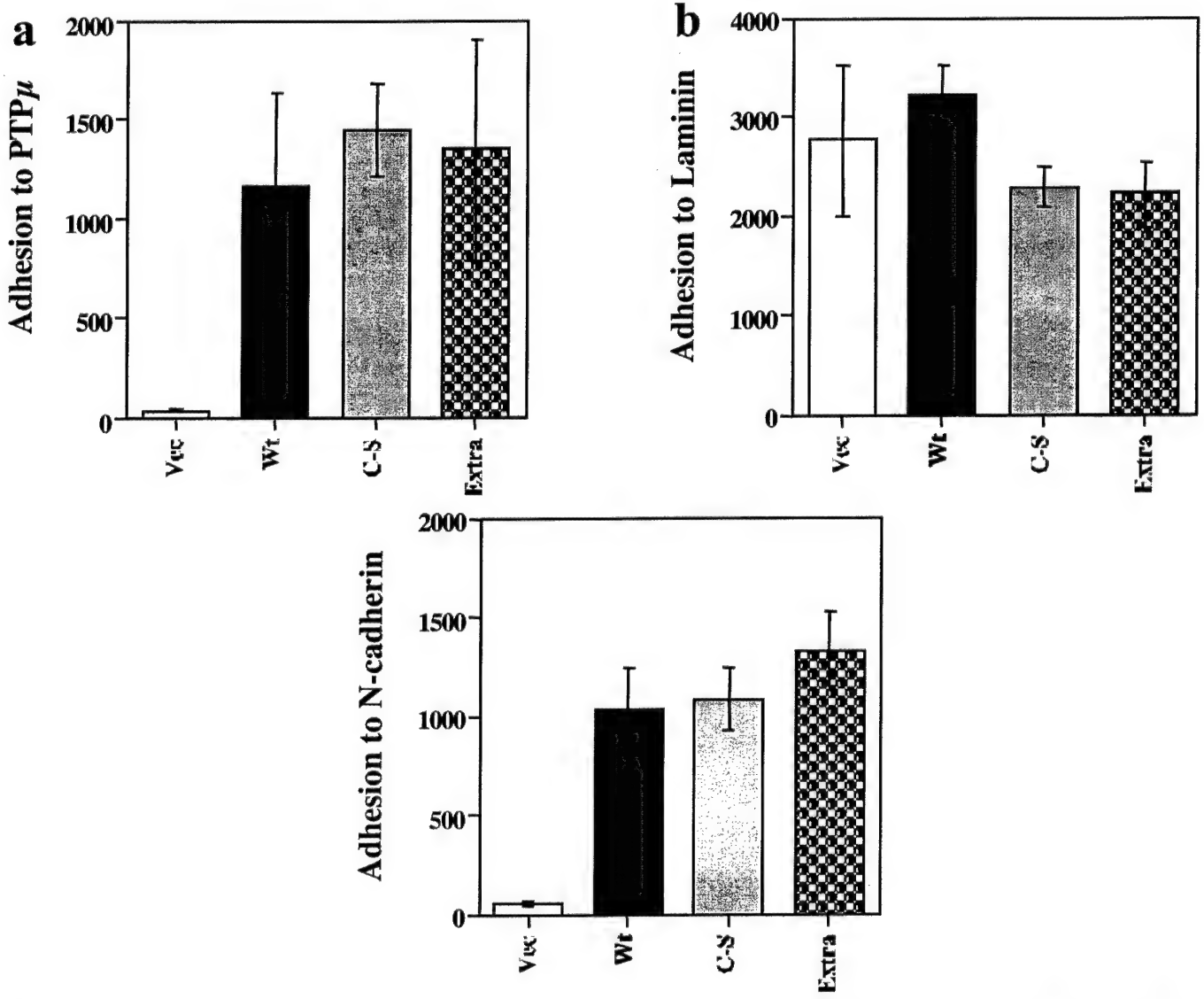


Fig.18. Re-expression of wild type, C-S and Extra PTPμ in LNCaP cells induced adhesion to N-cadherin. All forms of PTPμ induced adhesion to PTPμ as expected. Laminin adhesion was unaffected by PTPμ expression.

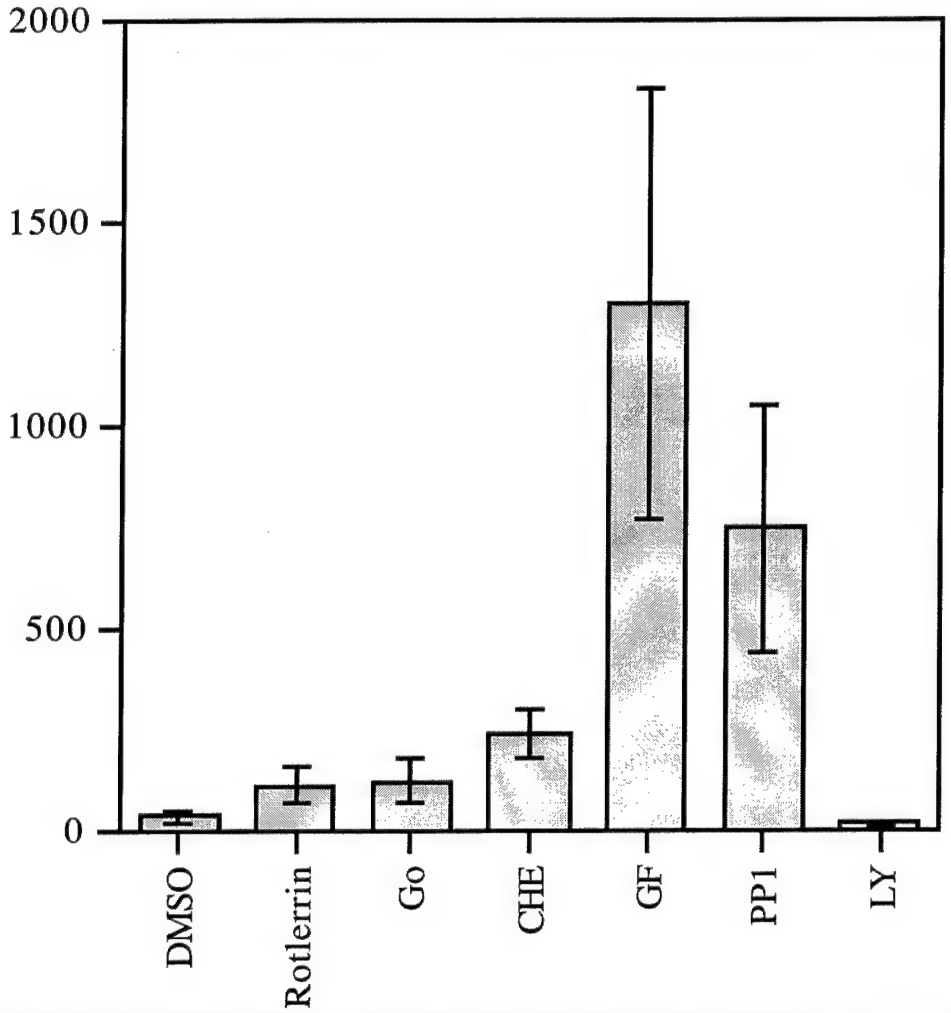


Fig. 19. Adhesion to N-cadherin is induced in parental LNCaP cells by inhibition of protein kinase C. The generic PKC inhibitor (GF) induce adhesion to E-cadherin. As did the Src inhibitor PP1. However, two other PKC inhibitors (CHE) and the PKCδ specific inhibitor, Rottlerrin (R) had no effect. These results indicate that PKCε inhibition may be involved in restoring adhesion to N-cadherin.

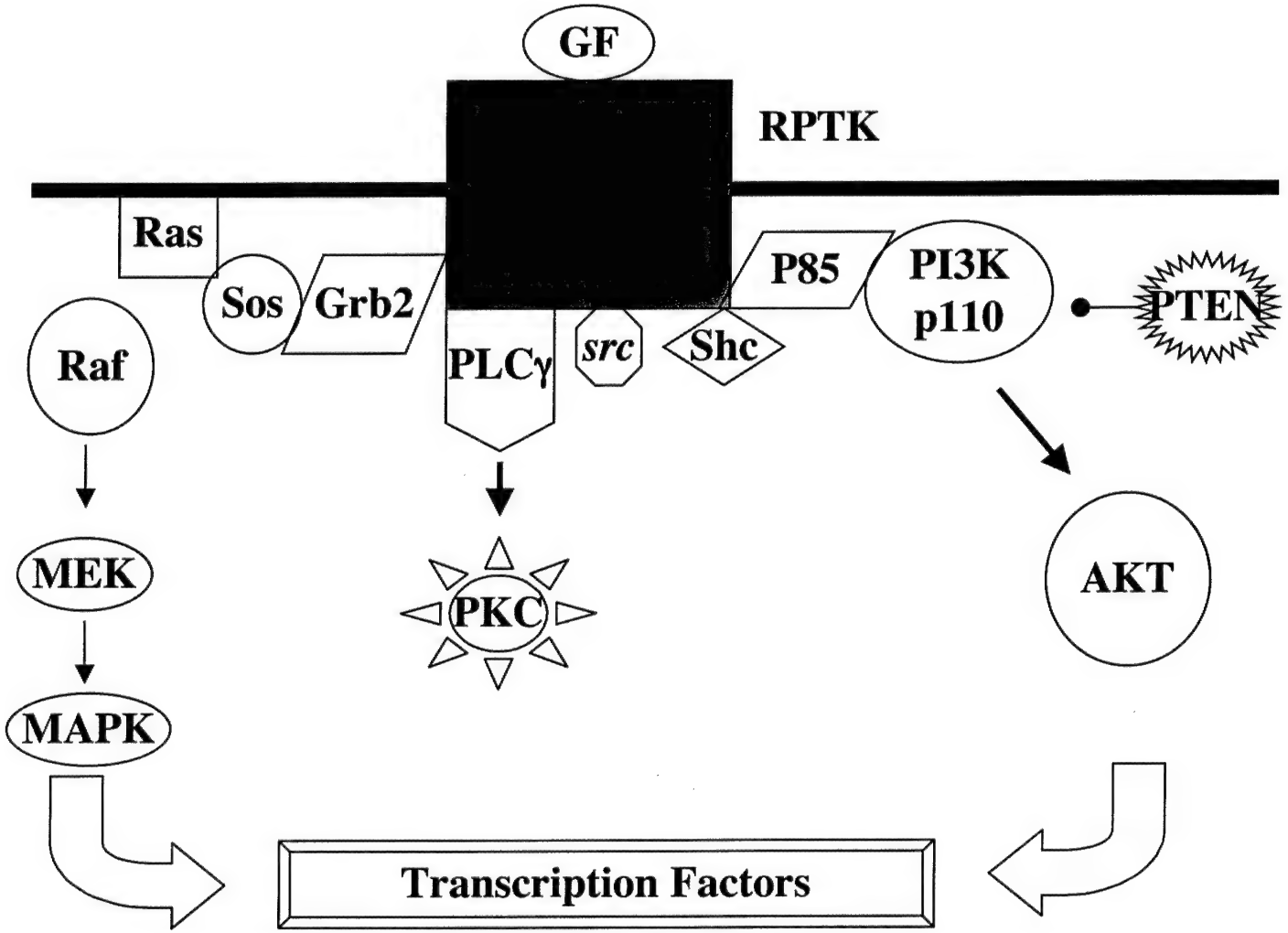


Fig. 20. Receptor protein tyrosine kinase signaling. Growth factors (GF) bind the receptor tyrosine kinase (gray rectangles) and activate tyrosine phosphorylation-dependent signals that recruit a number of proteins to activate the PI3K/AKT pathway as well as the Ras/MAPK pathway. The cellular signals ultimately result in activation of transcription factors.

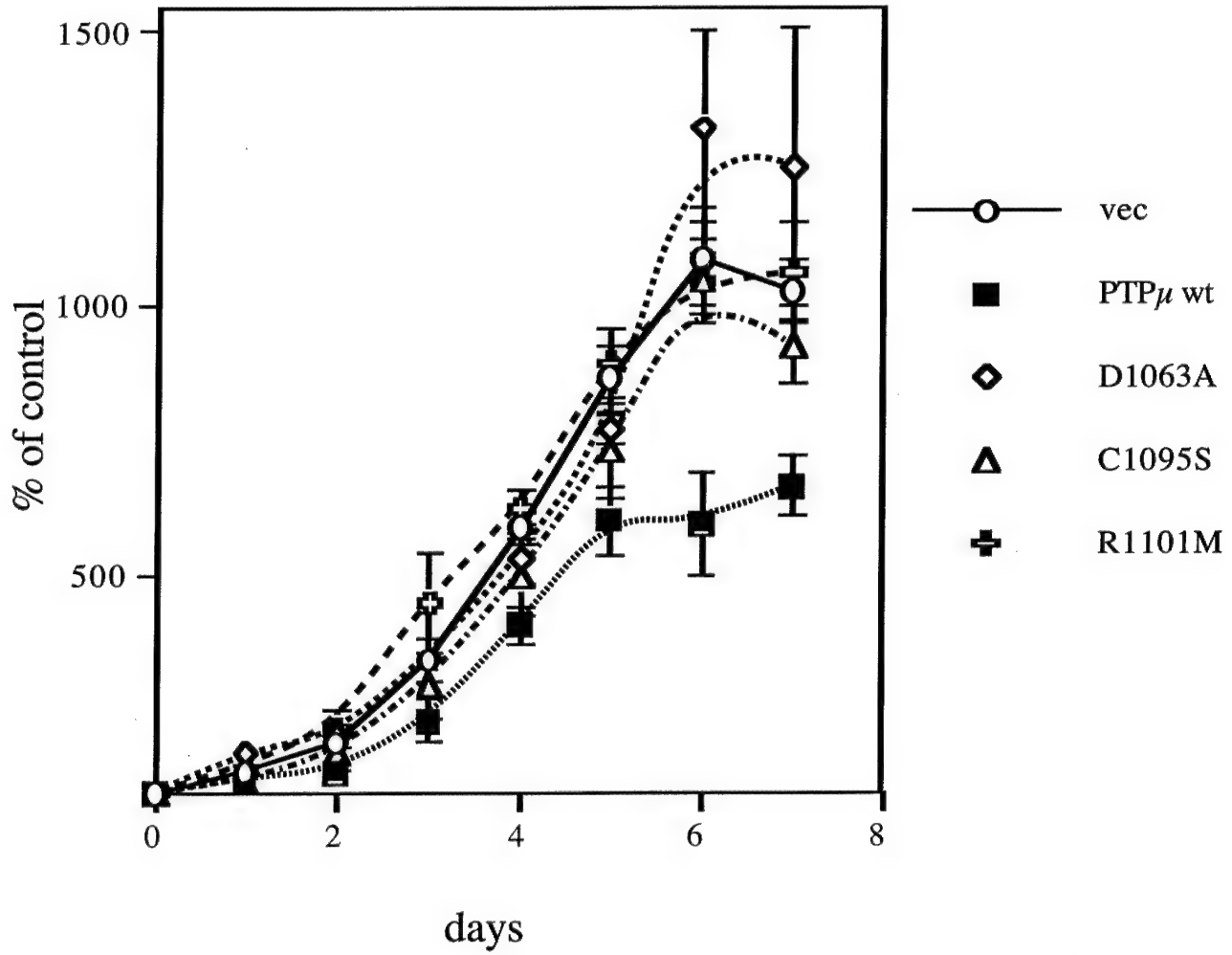


Fig. 21. Wild type PTPμ negatively regulated cell growth. Growth curves of infected cells are shown: Open circle is vector, black square is wild type PTPμ, open diamond D-A, open triangle C-S, and plus sign is R-M.

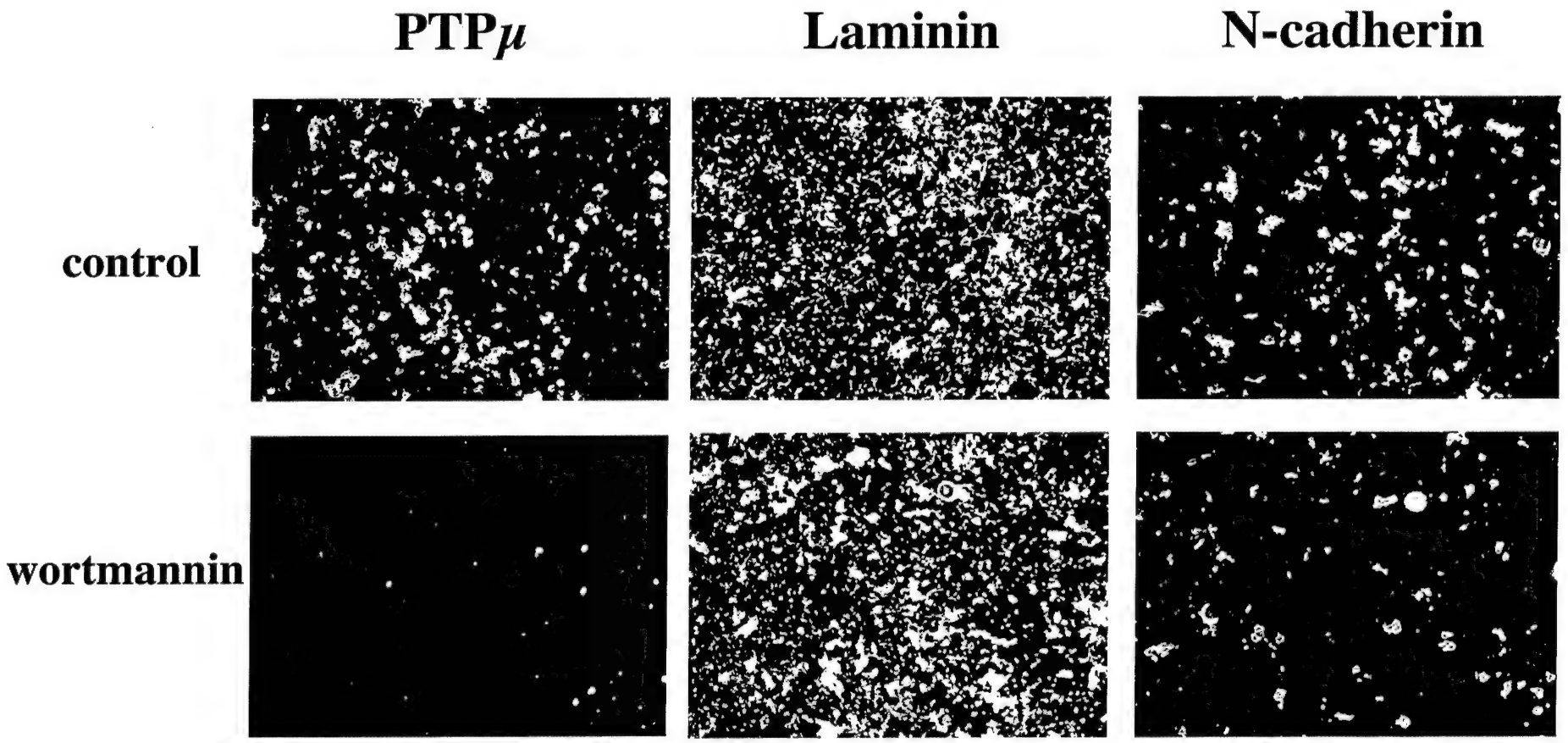


Fig. 22. PTPμ-dependent adhesion is selectively blocked by the PI3K inhibitor (wortmannin).

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 18, Issue of May 4, pp. 14896–14901, 2001
Printed in U.S.A.

The PTPμ Protein-tyrosine Phosphatase Binds and Recruits the Scaffolding Protein RACK1 to Cell-Cell Contacts*

Received for publication, November 30, 2000, and in revised form, February 9, 2001
Published, JBC Papers in Press, February 13, 2001, DOI 10.1074/jbc.M010823200

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PTPμ, an Ig superfamily receptor protein-tyrosine phosphatase, promotes cell-cell adhesion and interacts with the cadherin-catenin complex. The signaling pathway downstream of PTPμ is unknown; therefore, we used a yeast two-hybrid screen to identify additional PTPμ interacting proteins. The membrane-proximal catalytic domain of PTPμ was used as bait. Sequencing of two positive clones identified the scaffolding protein RACK1 (receptor for activated protein C kinase) as a PTPμ interacting protein. We demonstrate that RACK1 interacts with PTPμ when co-expressed in a recombinant baculovirus expression system. RACK1 is known to bind to the *src* protein-tyrosine kinase. This study demonstrates that PTPμ association with RACK1 is disrupted by the presence of constituitively active *src*. RACK1 is thought to be a scaffolding protein that recruits proteins to the plasma membrane via an unknown mechanism. We have shown that the association of endogenous PTPμ and RACK1 in a lung cell line is increased at high cell density. We also demonstrate that the recruitment of RACK1 to both the plasma membrane and cell-cell contact sites is dependent upon the presence of the PTPμ protein in these cells. Therefore, PTPμ may be one of the proteins that recruits RACK1 to points of cell-cell contact, which may be important for PTPμ-dependent signaling in response to cell-cell adhesion.

Control of tyrosine phosphorylation is regulated by the opposing actions of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs).[1] The PTP superfamily is a diverse group of proteins that include transmembrane receptors (1). Many of these receptor protein-tyrosine phosphatases (RPTPs) are members of the Ig superfamily, a group of proteins responsible for cell recognition or adhesion. We previously demonstrated that the RPTP PTPμ promotes cell-cell aggregation when expressed in nonadhesive cells (2–5). These studies demonstrated that the binding is homophilic (*i.e.* the "ligand" for PTPμ is an identical PTPμ molecule on an adjacent cell). Interestingly, endogenous levels of the PTPμ adhesion molecule have also been shown to promote neurite outgrowth from retinal neurons (6). RPTPs have cell adhesion molecule-like extracellular segments as well as intracellular domains possessing tyrosine phosphatase activity, suggesting they may play a regulatory role in cell adhesion-induced signaling (1, 7, 8). However, the precise signaling pathways utilized by RPTPs are unknown.

The juxtamembrane domain of PTPμ contains a region of homology to the conserved intracellular domain of the cadherins (9). Cadherins are calcium-dependent cell-cell adhesion molecules that interact with molecules termed catenins that associate with actin (10, 11). We previously demonstrated that PTPμ associates with a complex containing classical cadherins, α catenin and β catenin (12–14). In addition, we have recently demonstrated that PTPμ is required for *N*-cadherin-dependent neurite outgrowth (6). The signal transduction pathways downstream of the RPTPs and cadherins are not well understood. In this manuscript, we demonstrate that PTPμ interacts with RACK1 and that this protein may be a component of the PTPμ signaling pathway.

A recently identified group of cytosolic proteins called RACKs (receptors for activated protein C kinase) have been shown to bind to PKC only when it is in the activated state (15). It has been suggested that the binding of activated PKC to RACK(s) is necessary for the translocation of PKC to the plasma membrane, a process thought to be required in order for PKC to perform its physiological function (15). A specific RACK, RACK1, has been cloned and is a homolog of the β subunit of heterotrimeric G proteins as determined by the existence of WD repeats (16). WD repeats are 40-amino acid motifs proposed to mediate protein-protein interactions (17). RACK1 is composed of seven WD repeats that are thought to form propeller-like structures (18).

More recent data suggests that RACK1 is a scaffolding protein that recruits a number of signaling molecules into a complex. Theoretically, the seven propellers of the RACK1 structure (18) could bind seven different proteins. RACK1 has been shown to bind PKC, phospholipase Cγ, the *src* cytoplasmic protein-tyrosine kinase (PTK), cAMP-specific phosphodiesterase-4, the β subunit of integrins, and the β chain of interleukin-5 receptor (19–23). RACK1 has also been demonstrated to bind select pleckstrin homology domains *in vitro* including dynamin, β− spectrin, Ras GRF (guanine nucleotide-releasing factor), and oxysterol-binding protein (24). Some of the inter-

* This work was supported by a grant from the American Cancer Society, Ohio Division, Cuyahoga County Unit (to S. B. K.) and by National Institutes of Health Grant 1RO1-EY12251 (to S. B. K.). This work, under DAMD17-98-1-8586, was also supported by the Department of Defense Prostate Cancer Research Program, which is managed by the U.S. Army Medical Research and Materiel Command. Additional support was provided by Visual Sciences Research Center Core Grant from National Eye Institute Grant PO-EY11373. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by The Swedish Society for Medical Research.

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; RPTP, receptor PTP; PKC, protein kinase C; PTK, protein-tyrosine kinase; HA, hemagglutinin; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

actions between RACK1 and the proteins listed above have been shown to be mutually exclusive (20). In addition, only a subset of these interactions depend upon PKC stimulation (22). These studies suggest that RACK1 may form distinct signaling complexes in response to unique cellular stimuli.

In this study, we utilized the yeast two-hybrid genetic screen to isolate PTPμ interacting proteins and identified RACK1 as a protein that binds directly to the membrane-proximal catalytic domain in the cytoplasmic segment of PTPμ. We characterized this interaction using a recombinant baculovirus expression system and showed that RACK1 and PTPμ interact only when co-expressed. We also demonstrated that the presence of constituitively active *src* disrupts the interaction between PTPμ and RACK1. In MvLu cells, which endogenously express both PTPμ and RACK1, we demonstrated that PTPμ and RACK1 associate predominantly at higher cell densities. The association between RACK1 and PTPμ is not affected by activation of PKCs via phorbol esters. PTPμ is up-regulated at high cell density in MvLu cells (25) and is primarily found at cell-cell contact sites (12). We have found that RACK1 is recruited to the plasma membrane and points of cell-cell contact at high cell density. Antisense down-regulation of PTPμ expression results in a cytoplasmic localization of RACK1 even in the presence of cell contacts. Therefore, the recruitment of RACK1 to both the plasma membrane and cell-cell contact sites is dependent upon PTPμ. Localization of RACK1 to points of cell-cell contact may be an important part of the PTPμ-dependent signal transduction process in response to cell-cell adhesion.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—We used the LexA version of the yeast two-hybrid system to perform an interaction trap assay (26). This approach detects protein-protein interactions between a protein from a HeLa cell (human) cDNA library and a construct containing the membrane-proximal catalytic domain of human PTPμ (PTPμD1) as the bait. Amino acids 915–1178 (PTPμ-D1) were cloned in frame with the LexA coding sequence of pEG202 (*HIS3*) to generate a "bait" plasmid. The resulting construct (pEG202-D1) was sequenced for insertion and correct orientation. The pEG202-D1 plasmid and the β-galactosidase reporter plasmid (pSH18–34) were co-transformed into the yeast strain YPH499. The pSH18–34 (*URA3*) reporter plasmid contains a LexA-operator-*lacZ* fusion gene. The pEG202-D1 plasmid did not activate the β-galactosidase reporter plasmid on its own. A HeLa cell human cDNA library (26) in the pJG4–5 (*TRP1*) yeast expression vector was introduced into a yeast strain containing a chromosomal copy of the *LEU2* gene (EGY48), where the activating sequences of the *LEU2* gene are replaced with LexA operator sequences. The two strains (EGY48 and YPH499) were mated, and the resulting colonies containing the three plasmids were processed according to published methods (26). Potential interactions were detected by growing the mated yeast strain on minimal medium containing 2% galactose and 1% raffinose and lacking the appropriate amino acids to ensure selective pressure of the auxotrophic markers (only colonies containing all the plasmids and expressing the leucine reporter gene will grow). We screened 2.4×10^7 colonies and found four strong interactors. We isolated the prey-containing plasmids and sequenced the DNA from these clones. Two independent positive "prey" clones (clones 1 and 2) were identified as full-length RACK1 by DNA sequencing of the library plasmid (pJG4–5). The positive control used in Fig. 1*a* is a yeast strain containing a self-activating bait plasmid that grows on both glucose and galactose. The negative control for Fig. 1*b* is a yeast strain containing the bait (pEG 202-D1), reporter plasmid (pSH18–34), and an empty prey vector (pJG4–5). The positive control for Fig. 1*b* is Etk and HSP70 that interact with one another.[2]

A constituitively active form of the *src* protein-tyrosine kinase (Y527F,Y416F double mutant) was obtained from Dr. Jonathan Cooper (27). The BTM116 plasmid containing the *src* gene was restriction digested with *Bam*HI. A partial digest of pSH18–34 was performed with *Bam*HI, and the *src* insert was ligated with this vector. The pSH18–34/*src* and the pEG202-D1 plasmids were used to transform the YPH499 yeast strain. Then this YPH499 strain was mated to the EGY48 strain containing the RACK1 gene. This allowed us to test whether the *src* interaction with RACK1 could disrupt the RACK1/PTPμ interaction that drove β-galactosidase transcription.

Antibodies—Monoclonal antibodies against the intracellular (SK7, SK15, SK18) and extracellular (BK2) domains of PTPμ or polyclonal antibody against the intracellular domain (471) of PTPμ have been described (2, 5). A control monoclonal antibody directed against L1 (8D9) was generously provided by Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). A monoclonal antibody to the HA tag conjugated to horseradish peroxidase (Roche Molecular Biochemicals) was used to detect recombinant RACK1. The HA antibody was also purchased in a biotinylated form, and strepavidin-horseradish peroxidase was used for visualization (Covance, Denver, PA). In addition, a specific antibody to RACK1 was purchased from Transduction Labs (Lexington, KY). The monoclonal antibody to *src* was purchased from Calbiochem (San Diego, CA).

Baculoviruses—The baculovirus encoding the intracellular domain of PTPμ (intra-PTPμ) has been described (2). The *src* baculovirus, which encodes a constituitively active kinase (Y527F mutant), was kindly provided by Dr. Michael Weber and originally constructed by Dr. David Morgan (28). The RACK1-pJG4–5 vector was restriction digested so that the resulting 1800-base pair fragment contained an HA tag (from pJG4–5) in frame with the full-length RACK1 clone. The 1800-base pair fragment was cloned into the pAcHLT-C (BD Pharmingen, San Diego, CA) baculovirus expression vector. The construct was sequenced to confirm orientation and correct insertion of the 1800-base pair fragment. This created a form of RACK1 that contained a poly-histidine tag, a cAMP-dependent protein kinase site, a thrombin cleavage site, and an HA tag, at the N terminus followed by the RACK1 cDNA sequence (amino acids 1–317). Baculoviruses were generated using the BaculoGold™ system (BD PharMingen).

Expression in Insect Cells—Sf9 cells (CRL 1711; American Type Culture Collection, Manassas, VA) derived from the ovary of the Fall army worm *Spodoptera frugiperda* were maintained at 27 °C in Grace's Insect Medium Supplemented (Life Technologies, Inc.) containing 10% fetal bovine serum and 10 μg/ml gentamicin. The viral stocks were then used to infect Sf9 cells and express the proteins of interest (PTPμ, RACK1, and *src*). 48 h post-infection, cells were either lysed or treated with 160 nM PMA (activator of PKC; Calbiochem) (22) or PP2 (inhibitor of *src* family kinases; Calbiochem) for 30 min at 27 °C. Cells were lysed in ice-cold buffer (1% Triton X-100, 20 mM Tris, pH 7.6, 1 mM benzamidine, 2 μl/ml protease inhibitor mixture (Sigma), 150 mM NaCl, 0.2 μM okadaic acid, 200 μM phenylarsine oxide, 1 mM vanadate, and 0.1 mM molybdate) for 30 min on ice. The lysates were centrifuged at $3,000 \times g$ for 3 min to remove Triton-insoluble components.

MvLu Cell Experiments—Mv 1 Lu (MvLu) mink lung epithelial cells (ATCC number CCL 64) were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium containing 10 μg/ml gentamicin plus 10% fetal bovine serum (Life Technologies, Inc.). The MvLu cells were grown to 50 or 90% confluence prior to lysis. PKC activation of MvLu cells was performed by adding 10 nM PMA (Calbiochem) for 15 min at 37 °C before lysis of the cells. MvLu cells were lysed in buffer (20 mM Tris, pH 7.6, 1% Triton X-100, 2 μl/ml protease inhibitor mixture, 1 mM benzamidine, 200 μM phenyl arsine oxide, 1 mM vanadate, and 0.1 mM molybdate), incubated on ice for 30 min, and centrifuged at $14,000 \times g$ for 3 min.

A replication-defective amphotrophic retrovirus expressing an antisense PTPμ construct and control retrovirus have been described previously (6). MvLu cells were incubated in virus-containing medium supplemented with serum (final concentration, 10% final) plus 5 μg/ml polybrene for 4 days. Reduction in endogenous PTPμ expression was verified by immunoblotting lysates from infected cells with antibodies to PTPμ.

Immunoprecipitations and Electrophoresis—For immunoprecipitation, antibodies to PTPμ or RACK1 were incubated with protein A-Sepharose (Amersham Pharmacia Biotech) or goat anti-mouse IgG (or IgM) that had been conjugated to Sepharose (Zymed Laboratories Inc., South San Francisco, CA) for 2 h at room temperature then washed three times with phosphate-buffered saline (9.5 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.5) before addition to cell lysates. Purified monoclonal antibodies were used at 0.6 mg of IgG/ml beads, ascites fluid was used at 1 mg of IgG/ml beads, and polyclonal serum was used at 3 mg of IgG/ml beads. Immunoprecipitates were prepared from 40 μg (Sf9 cells) or 300 μg (MvLu cells) of a Triton-soluble lysate of cells as measured by the Bradford method. The immunoprecipitates were incubated overnight at 4 °C on a rocker and centrifuged at $3,000 \times g$ for 1 min. The supernatant was removed from the beads, the beads were washed four times in lysis buffer, and the bound material eluted by

[2] H. J. Kung and Y. Qiu, personal communication.

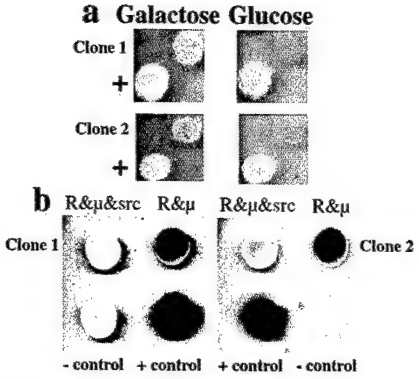

FIG. 1. **PTPμ and RACK1 interact in yeast.** *a* illustrates the growth of two yeast clones containing both RACK1 (*R*) and PTPμ (*μ*) grown on medium containing galactose or glucose. *b* shows the same PTPμ/RACK1 (*R&μ*) clones grown on medium containing X-gal. Positive (+) and negative (−) controls are described under "Experimental Procedures." The yeast strain containing PTPμ, RACK1, and *src* (*R&μ&src*) is also shown.

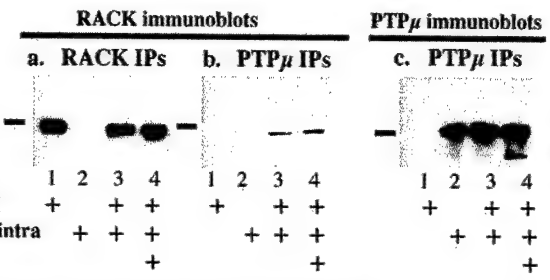

FIG. 2. **PTPμ and RACK1 interact in Sf9 cells.** *a–c* are immunoprecipitations that were separated by 7.5% or 10% SDS-PAGE and probed with monoclonal antibodies to either the HA tag of RACK1 (*a* and *b*) or intracellular domain of PTPμ (*c*). *a* demonstrates that equal amounts of RACK1 are present in RACK1 immunoprecipitates from Sf9 cells infected with either RACK1 (*lane 1*), RACK1 and PTPμ (*lane 3*), RACK1/PTPμ plus PMA (*lane 4*). Cells infected with PTPμ only (*lane 2*) display no detectable RACK1. *b* illustrates immunoblots of PTPμ immunoprecipitates probed with HA antibody to detect the RACK1 protein. PTPμ and RACK1 interact only when they are co-expressed regardless of the presence or absence of PMA (*lanes 3* and *4*). The *bar* to the *left* of *a* and *b* represents the 52-kDa molecular mass marker. Recombinant RACK1 migrates at this molecular mass because of the addition of various tags. *c* shows an immunoblot of PTPμ immunoprecipitates that were separated by 7.5% SDS-PAGE and probed with a monoclonal antibody to PTPμ (SK15). In all Sf9 cells infected with PTPμ, equal amounts of PTPμ are present. Cells infected with RACK1 only (*lane 1*) display no detectable PTPμ. The *bar* in *c* represents the 91-kDa molecular mass marker.

addition of 100 μl of 2× sample buffer and heated for 5 min at 95 °C. One-fifth of the immunoprecipitate (20 μl) was loaded per lane of the gel, and the proteins were separated by 10% (for analysis of RACK1 and *src*), 7.5% (for analysis of the intracellular PTPμ), and 6% (for analysis of endogenous PTPμ) SDS-PAGE and transferred to nitrocellulose for immunoblotting as previously described (2).

Immunocytochemistry—All chemicals were diluted in phosphate-buffered saline. Cells were fixed with 2% paraformaldehyde for 30 min. at room temperature (Electron Microscopy Sciences, Fort Washington, PA). The cells were permeabilized with 0.5% saponin, blocked with 20% normal goat serum plus 1% BSA and incubated with primary antibody for 18 h at 4 °C. The cells were washed with TNT buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween 20) containing 0.5% saponin and incubated with Texas Red, fluorescein, or rhodamine-conjugated secondary antibody (Molecular Probes, Eugene, OR or Cappel Research Products, Durham, NC) for 1.5 h at room temperature. Samples were mounted with Slow fade Light (Molecular Probes). The fluorescent labeling was examined using a 40× objective on a Zeiss Axioplan 2 microscope equipped for epifluorescence. Images were captured using a Hamamatsu cooled CCD camera.

RESULTS

Yeast Two-hybrid Analysis—The yeast two-hybrid interaction trap assay (26) was used to identify proteins that were capable of binding directly to the membrane-proximal catalytic domain of PTPμ. Potential interactors were detected by growing the mated yeast strain on minimal medium containing 2% galactose and 1% raffinose and lacking the appropriate amino acids (only colonies containing all the plasmids and expressing the leucine reporter gene will grow). We screened 2.4×10^7 colonies. PTPμ interactors were selected by three criteria. First, they were screened for viability on medium lacking leucine. Only interacting clones will be able to grow on medium without leucine. Second, they were screened for formation of blue colonies when grown on medium containing X-gal/galactose compared with X-gal/glucose containing medium. Galactose specifically induces expression of the "prey" protein whereas glucose does not. Third, they were discriminated for the level of transcriptional activation of the *lacZ* gene based on the blue color of the colonies when grown on medium containing X-gal. Fig. 1 illustrates two independent clones (clones 1 and 2) that fulfilled these criteria. The two clones did not grow on glucose but did grow on galactose (Fig. 1*a*). These two clones also expressed high levels of β-galactosidase (Fig. 1*b*, *R&μ*). Sequence analysis of these two independent positive clones demonstrated that they both encoded RACK1, a member of the heterotrimeric G_β superfamily of proteins.

RACK1 and PTPμ Interact in Sf9 Cells—To characterize the interaction of PTPμ and RACK1, Sf9 cells were infected with baculoviruses encoding full-length RACK1 or the intracellular domain of PTPμ, singly or in combination. Lysates from Sf9 cells were immunoprecipitated with RACK1 or PTPμ (471) antibodies and resolved by SDS-PAGE. Immunoblots of immunoprecipitates probed with an antibody to RACK1 are shown (Fig. 2, *a* and *b*). Immunoblots of PTPμ immunoprecipitates probed with an anti-PTPμ (SK15) antibody are shown (Fig. 2*c*). Equal amounts of RACK1 were available in the Triton-soluble lysate used for immunoprecipitation based on the ability of RACK1 antibodies to immunoprecipitate RACK1 (Fig. 2*a*, *lanes 1*, *3*, and *4*). Fig. 2 also illustrates that equal amounts of PTPμ were immunoprecipitated from the PTPμ-infected cell samples (Fig. 2*c*, *lanes 2–4*). PTPμ immunoprecipitates (471 antibody) were immunoblotted with antibody to RACK1 (Fig. 2*b*). RACK1 interaction with PTPμ was only detected in cells where both proteins were co-expressed (Fig. 2*b*, *lanes 3* and *4*). The PTPμ antibody did not immunoprecipitate RACK1 from cell lysates in the absence of PTPμ expression (Fig. 2*b*, *lane 1*), thus ruling out the possibility that the antibody recognized RACK1 nonspecifically. Treatment with phorbol esters can stimulate PKC because of their close chemical similarity to diacylglycerol (15). An endogenous PKC that resembles the nonconventional PKCs is expressed in Sf9 cells and is known to be stimulated 20-fold by phorbol ester treatment (29). The interaction between RACK1 and PTPμ was unaffected by endogenous PKC stimulation with phorbol esters (Fig. 2*b*, *lane 4*). These data indicate that RACK1 only bound to PTPμ when both proteins were co-expressed in insect cells and the interaction was not affected by phorbol esters.

src Disrupts the Interaction between RACK1 and PTPμ—Because RACK1 is known to bind to *src* (20), we tested whether addition of a constituitively active *src* to the yeast cells affected binding between RACK1 and PTPμ. β-Galactosidase staining of yeast expressing RACK1 and PTPμ was positive, whereas RACK1/PTPμ/*src* containing yeast did not turn blue on medium containing X-gal (Fig. 1*b*). Therefore, constituitively active *src* appears to disrupt the interaction between RACK1 and PTPμ in yeast.

We then analyzed whether constituitively active *src* could disrupt the interaction between RACK1 and PTPμ in the Sf9 cell system. We did single, double, or triple infections with

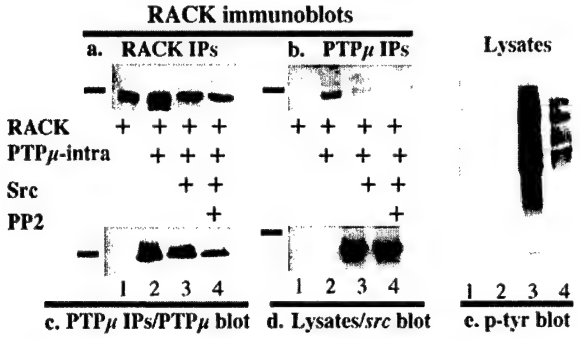

FIG. 3. **The interaction between RACK1 and PTPμ is disrupted by constituitively active *src*.** Immunoprecipitates are shown from Sf9 cells infected with either RACK1 (*lane 1*), RACK1 and PTPμ (*lane 2*), RACK1/PTPμ/*src* (*lane 3*), and RACK1/PTPμ/*src* in the presence of the *src* tyrosine kinase inhibitor PP2 (*lane 4*). *a* demonstrates that equal amounts of RACK1 are present in RACK1 immunoprecipitates. *b* illustrates immunoblots of PTPμ immunoprecipitates probed with HA antibody to detect the RACK1 protein. *c* shows an immunoblot of PTPμ immunoprecipitates that were separated by 7.5% SDS-PAGE and probed with a monoclonal antibody to PTPμ (SK15). An immunoblot of lysates using a *src* antibody demonstrates that *src* is expressed only in cells infected with the *src* virus (*d*). An immunoblot of lysates using an anti-phosphotyrosine antibody demonstrates that *src* is inhibited in cells infected with the *src* virus in the presence of the PP2 *src* inhibitor (*e*).

RACK1, PTPμ, and constituitively active *src*. Fig. 3 shows that PTPμ (Fig. 3*c*) and the *src* PTK (Fig. 3*d*) were expressed in the appropriate samples. RACK1 antibody immunoprecipitated RACK1 from all the appropriate samples (Fig. 3*a*, *lanes 1–4*). PTPμ immunoprecipitated PTPμ in all the appropriate samples (Fig. 3 c). Although RACK1 and PTPμ interact when co-infected (Fig. 3*b*, *lane 2*), there was no interaction detected when *src* was added in the triple infection (Fig. 3*b*, *lanes 3* and *4*). PP2, the cell-permeable *src* family kinase inhibitor, had no effect on the ability of *src* to disrupt the PTPμ/RACK1 interaction (Fig. 3*b*, *lane 4*). PP2 was able to inhibit *src* tyrosine kinase activity as evidenced by a decrease in anti-phosphotyrosine reactivity (Fig. 3*e*). These results suggest that the ability of *src* to disrupt the PTPμ/RACK1 interaction was not dependent upon kinase activity or mediated by tyrosine phosphorylation of any of the proteins. Therefore, constituitively active *src* was able to disrupt the interaction between RACK1 and PTPμ in yeast as well as in the Sf9 cell system. Together, these results suggest that PTPμ and *src* may form mutually exclusive complexes with RACK1.

Endogenous RACK1 Interacts with Endogenous PTPμ—To examine whether endogenous PTPμ associates with endogenous RACK1, immunoprecipitation experiments were performed using MvLu cells. Endogenous PTPμ in MvLu cells is proteolytically processed. The full-length form is cleaved into two noncovalently associated fragments, one (P-subunit) comprising the entire intracellular and transmembrane segments and a short stretch of extracellular sequence, the other (E-subunit) containing the remainder of the extracellular segment (5, 25). Both the full-length (200 kDa) and cleaved form (100 kDa) of PTPμ were expressed (Fig. 4*a*) as expected (12). PTPμ expression in MvLu cells is regulated by cell density (25). In our studies, the expression of PTPμ also increased with cell density (data not shown). However, at 50 and 90% confluence, PTPμ expression appeared to be approximately the same (Fig. 4*a*). MvLu cell cultures were grown at these two densities to control the amount of cell contact. When MvLu cells are grown to 50% confluence there is little cell contact, whereas at 90% confluence the majority of cells adhere to one another.

We used a retrovirus encoding antisense PTPμ (6) to infect MvLu cells. Immunoblot analysis demonstrated that the full-length protein (200-kDa band) was substantially reduced in cells infected with PTPμ antisense virus when compared with cells infected with control virus (Fig. 4*a*, *lane 5*). The 100-kDa immunoreactive band was also reduced (Fig. 4*a*, *lane 5*). These results confirmed that PTPμ antisense expression inhibited the new synthesis of PTPμ.

RACK1 was immunoprecipitated by antibodies to RACK1 (Fig. 4*c*), but was not detected when a control mouse antibody was used for immunoprecipitation (Fig. 4*b*). PTPμ was immunoprecipitated with a monoclonal antibody to the extracellular domain (BK2; Fig. 4*d*) or with a polyclonal antibody to the C terminus (471; Fig. 4*e*). When immunoprecipitates of PTPμ were probed on immunoblots with anti-RACK1 antibody, an association was detected (Fig. 4, *d* and *e*). The association increased at high cell density (Fig. 4, *d* and *e*, *lane 3*). The interaction was not substantially altered when PKCs were stimulated by phorbol ester (PMA) treatment (Fig. 4, *d* and *e*, *lane 4*). However, when PTPμ expression was reduced in antisense-infected cells, it no longer interacted with RACK1 (Fig. 4, *d* and *e*, *lane 5*). These data suggest that endogenous RACK1 and PTPμ interact predominantly when cell-cell contact occurs.

RACK1 Localizes to Points of Cell-Cell Contact at High Cell Density—PTPμ localizes to points of cell-cell contact in MvLu cells (12) and as shown in Fig. 5 (*a–d*). Immunocytochemical analysis of subconfluent cultures of MvLu cells is shown in Fig. 5 (*a* and *b*). PTPμ localized to filopodial extensions that contacted between adjacent MvLu cells (Fig. 5, *a* and *b*). When cells were plated at higher density, PTPμ was restricted to points of cell-cell contact (Fig. 5, *c* and *d*). To determine the localization of RACK1, we performed immunocytochemistry on MvLu cells. The RACK1 protein is predominantly cytoplasmic in subconfluent MvLu cells (Fig. 5, *e* and *f*). As cell density increased RACK1 translocated to the plasma membrane. At high cell density, RACK1 also decorated points of cell-cell contact in MvLu cells (Fig. 5, *g* and *h*). We performed double-label immunocytochemistry on MvLu cells using antibodies to PTPμ (Fig. 6*b*) and RACK1 (Fig. 6*c*). The *arrows* in Fig. 6 illustrate concentration of RACK1 and PTPμ at sites of cell-cell contact. The translocation of RACK1 to the membrane and points of cell-cell contact at high cell density is likely to be related to its increased association with PTPμ (Fig. 4).

We then used a retrovirus encoding antisense PTPμ to infect MvLu cells and performed immunocytochemistry. There was a dramatic morphological change in the MvLu cells when infected with the antisense PTPμ retrovirus (Fig. 7). The antisense infected cells were never able to grow to high cell density. However, cell-cell contact sites were still present as evidenced by localization of cadherins (Fig. 7, *a* and *b*). When PTPμ expression was reduced, RACK1 no longer localized to the plasma membrane or points of cell-cell contact (Fig. 7, *c* and *d*). These data suggest that the PTPμ protein plays a role in recruiting RACK1 to points of cell-cell contact in MvLu cells.

DISCUSSION

In this study, we used the yeast two-hybrid screen to isolate PTPμ interacting proteins. We identified an interaction between the membrane-proximal catalytic domain of PTPμ (PTPμD1) and RACK1. Because yeast do not have traditional tyrosine kinases, the interaction of PTPμD1 and RACK1 was likely to be mediated by protein-protein interactions and not dependent upon phosphotyrosine. We characterized the association between RACK1 and PTPμD1 using the recombinant baculovirus expression system and have shown that the intracellular segment of PTPμ binds to RACK1 in insect cells. The RACK1/PTPμ interaction was disrupted by a constituitively active *src* PTK. These data suggest that RACK1/*src* and RACK1/PTPμ may form mutually exclusive signaling complexes. In addition, we showed an association between PTPμ

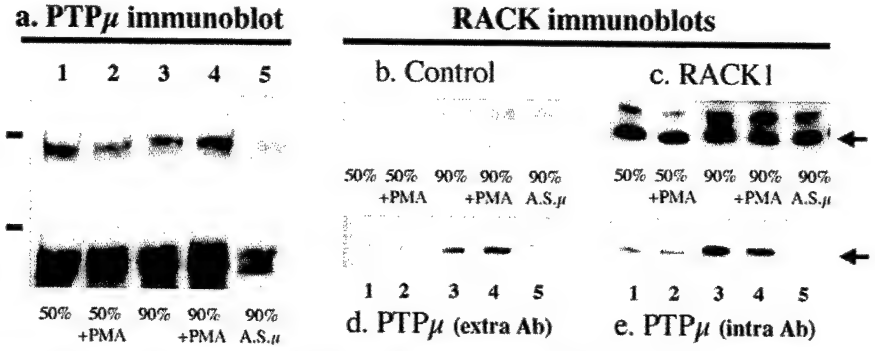

FIG. 4. **Endogenous RACK1 interacts with endogenous PTPμ and the interaction is not altered by PMA treatment.** MvLu cells were grown to 50% (*lanes 1* and *2*) or 90% confluence (*lanes 3–5*) or MvLu cells were treated with phorbol esters (PMA, *lanes 2*, and *4*). Lysates from MvLu cells were immunoprecipitated with control (*b*), RACK1 (*c*), or PTPμ (*d* and *e*) antibodies as indicated and were separated by 10 or 6% SDS-PAGE. *a* is an immunoblot of lysates using an antibody to PTPμ (SK15). *b–e* are immunoblots of the immunoprecipitates probed with antibody to RACK1. The control antibody (8D9) did not immunoprecipitate RACK1 (*b*). RACK1 was readily detectable in all RACK1 immunoprecipitates (*c*). PTPμ interacted with RACK1 predominantly in 90% confluent MvLu cells (*d* and *e*, *lanes 3* and *4*). Importantly, the interaction was not substantially altered by PMA treatment (*d* and *e*, *lane 4*). Finally, the interaction between PTPμ and RACK1 was abolished by down-regulating PTPμ expression using a virus encoding antisense PTPμ (A.S.μ) (*d* and *e*, *lane 5*). The molecular mass markers in *a* represent the 208- and 130-kDa markers, respectively. The *arrows* in each panel indicate the 36-kDa RACK1 band.

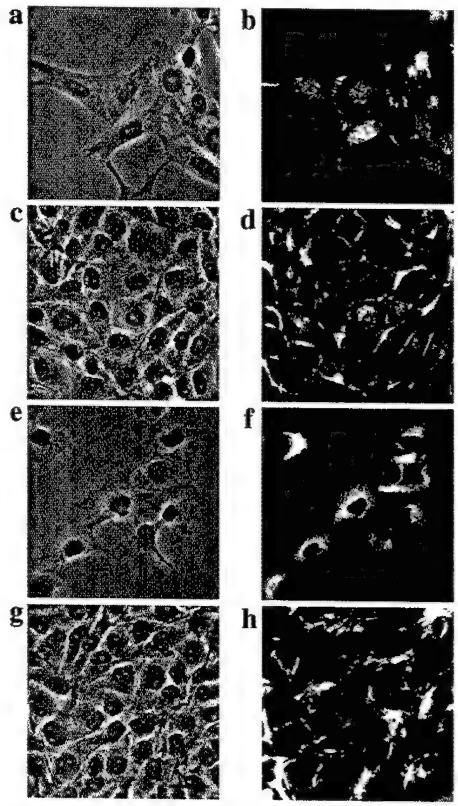

FIG. 5. **Immunocytochemical localization of PTPμ and RACK1 in MvLu cells.** Phase contrast (*a*, *c*, *e*, and *g*) or fluorescence (*b*, *d*, *f*, and *h*) images of MvLu cells are shown. Cells labeled with antibodies to PTPμ (SK15, *a–d*) show that PTPμ is localized to filopodial extensions of the cells and points of cell-cell contact in subconfluent MvLu cells (*a* and *b*). When the cells were plated at higher density, PTPμ was localized to points of cell-cell contact (*c* and *d*). RACK1 (*e–h*) was localized in the cytoplasm at low cell density (*e* and *f*). At high cell density, RACK1 was localized to points of cell-cell contact (*g* and *h*). *Scale bar*, 30 μm.

FIG. 6. **Double-label immunocytochemistry of RACK1 and PTPμ in confluent MvLu cells.** Phase contrast (*a*) or fluorescence (*b* and *c*) images of MvLu cells are shown. Cells labeled with antibodies to PTPμ (*b*) show that PTPμ is localized points of cell-cell contact. RACK1 (*c*) was also localized to points of cell-cell contact. *Arrows* indicate some of the areas of co-localization. *Scale bar*, 20 μm.

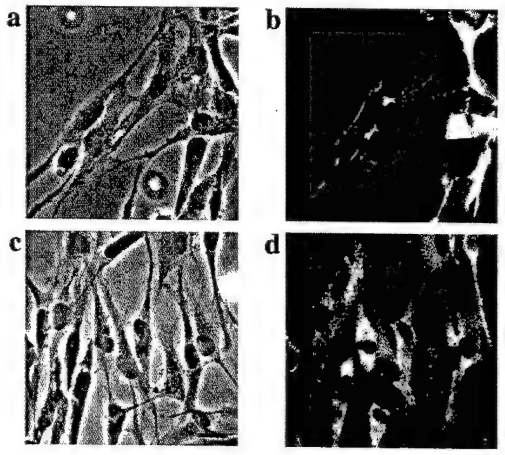

FIG. 7. **Immunocytochemical localization of cadherins and RACK1 in MvLu cells infected with an antisense PTPμ-encoding retrovirus.** A retrovirus encoding antisense PTPμ was used to infect MvLu cells and immunocytochemistry was performed. When PTPμ expression was reduced, some cell-cell contacts sites were still present as evidenced by localization of classical cadherins using the pan cadherin antibody (*a* and *b*). However, RACK1 no longer localized to the plasma membrane and points of cell-cell contact (*c* and *d*). These data suggest that the PTPμ protein plays a role in recruiting RACK1 to points of cell-cell contact in MvLu cells. *Scale bar*, 20 μm.

and RACK1 using MvLu cells, which express both proteins endogenously. The interaction of PTPμ and RACK1 was not affected by phorbol ester stimulation of PKC, suggesting that when RACK1 is bound to PTPμ it is still likely to be able to bind PKC. PTPμ expression increases with increasing cell density in MvLu cells (25). At high cell density, we observed an increased association of RACK1 with PTPμ as well as increased translocation of RACK1 to the plasma membrane and points of cell-cell contact. In cells expressing antisense PTPμ, RACK1 remained cytoplasmic, suggesting that PTPμ may play a role in recruiting RACK1 to cell contact sites. Together these data suggest that PTPμ and RACK1 form a signaling complex at high cell density.

RACK1 is a homolog of the β subunit of heterotrimeric G proteins and is composed of WD repeats (16). Both G_β and RACK1 form seven propeller structures (seven independently

folding loops) proposed to mediate protein-protein interactions (17). RACK1 was originally identified as a protein that binds to activated PKC (16). It has been suggested that activated PKC binding to RACK1 is required for the translocation of the enzyme to the plasma membrane, its physiologically relevant site of action (15). In addition, RACK1 seems to serve as a general scaffolding protein for a number of signaling enzymes including *src* (20).

Receptor protein-tyrosine phosphatases are involved in cell adhesion (1). PTPμ has been shown to induce cell adhesion by homophilic binding (2, 3, 5). In addition, it also appears to regulate cadherin-mediated cell adhesion by binding to the cadherin/catenin complex (6, 12, 13). The PTPμ/RACK1 interaction appears to occur predominantly in cells at high cell density. These data indicate that the PTPμ/RACK1 interaction may be induced by cell-cell contact. Based on our antisense experiments, it is clear that the interaction requires the presence of the PTPμ protein. Our hypothesis is that the PTPμ/RACK1 interaction is likely to be induced by cell adhesion, which may recruit other signaling proteins that are important for PTPμ-dependent signal transduction. One could speculate that PKC or other signaling molecules associated with RACK1 might be downstream of PTPμ-dependent signals induced by cell-cell adhesion.

If the association of RACK1 and PTPμ brings activated PKC close to its site of action at the membrane, how might other cell-cell adhesion molecules, like cadherins, be involved? There have been suggestions in the literature that PKC may regulate E-cadherin-dependent adhesion. Adherens junctions serve to anchor the actin cytoskeleton at regions of cell-cell contact. Investigators have postulated that the cytoskeletal reorganization that occurs during the formation of adherens junctions is induced by PKC activation and that PKC, in turn, may regulate cadherin-dependent adhesion (30). Clearly our data suggest that an interesting relationship exists between PKC signal transduction mechanisms and the PTPμ cell-cell adhesion molecule. It is likely that the interaction of PTPμ and RACK1 at high cell density recruits PKC or other RACK1 binding partners to sites of cell-cell contact to transduce adhesion-dependent signals.

Tyrosine phosphorylation by the *src* PTK negatively regulates cadherin-dependent adhesion (7, 31, 32); although the mechanism is unknown. Previously, we tested the ability of the *src* tyrosine kinase to regulate PTPμ/cadherin interactions. We used a series of WC5 cell lines that express PTPμ endogenously and ectopically expressed E-cadherin. We analyzed the effect of tyrosine phosphorylation on the composition of the PTPμ-cadherin complex, and our data suggested that increased tyrosine phosphorylation of E-cadherin resulted in decreased association with PTPμ (13). Interestingly, RACK1 binds to the *src* PTK. In this study, we found that constituitively active *src* disrupts the binding between PTPμ and RACK1. Because *src* is known to negatively regulate cadherin-dependent adhesion, the ability of PTPμ and *src* to bind RACK1 may directly affect tyrosine phosphorylation of the cadherin complex via PTPμ or indirectly by regulating the presence of the *src* PTK in the complex. Together, these data suggest that PTPμ may be altering *src* signaling pathways via its interaction with RACK1.

Because RACK1 binds to the conserved PTP catalytic domain of PTPμ, a number of other PTPs may also interact with RACK1. It is interesting to note that many PTPs are known to regulate the *src* cytoplasmic PTK (1). Importantly, RACK1 is known to bind to c-*src* and inhibit its tyrosine kinase activity (20). Based upon the results from that study, c-*src* does not appear to phosphorylate native RACK1 (20). Our data suggest that one of the links between PTPs and *src* PTK signaling may be the RACK1 protein. This manuscript demonstrates that PTPμ association with RACK1 is altered in the presence of *src*,• suggesting that there may be mutually exclusive interactions of *src* and PTPμ with RACK1. One caveat of these studies is that they were done using a constituitively active *src* PTK. However, we believe that the PTP *versus* PTK competition for binding to RACK1 may be a common form of regulation for signaling complexes. For example, protein-protein interactions with scaffolding molecules such as RACK1 may control tyrosine phosphorylation of substrate proteins via their mutually exclusive interactions with a tyrosine kinase or phosphatase. More importantly, the ability of *src* and PTPμ to compete for RACK1 binding may be an important mechanism for regulation of cell-cell adhesion and signal transduction.

Acknowledgments—A number of individuals provided assistance with this study, and their efforts were greatly appreciated, including Dr. Leif Stordal, Rachna Dave, Carol Luckey, Dr. Sandra Lemmon's lab and Dr. Hsing Jien Kung's lab especially L. Ravi. We would also like to thank Dr. Vance Lemmon, Dr. Hsing Jien Kung, Dr. Steven Reeves, Dr. Jonathan Cooper, Dr. Michael Weber, and Dr. David Morgan for reagents. We also thank Dr. Carole Leidtke for reagents and helpful suggestions.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 277, No. 13, Issue of March 29, pp. 11165–11173, 2002
Printed in U.S.A.

Expression of the Receptor Protein-tyrosine Phosphatase, PTPμ, Restores E-cadherin-dependent Adhesion in Human Prostate Carcinoma Cells*

Received for publication, December 19, 2001, and in revised form, January 17, 2002
Published, JBC Papers in Press, January 18, 2002, DOI 10.1074/jbc.M112157200

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Normal prostate expresses the receptor protein-tyrosine phosphatase, PTPμ, whereas LNCaP prostate carcinoma cells do not. PTPμ has been shown previously to interact with the E-cadherin complex. LNCaP cells express normal levels of E-cadherin and catenins but do not mediate either PTPμ- or E-cadherin-dependent adhesion. Re-expression of PTPμ restored cell adhesion to PTPμ and to E-cadherin. A mutant form of PTPμ that is catalytically inactive was re-expressed, and it also restored adhesion to PTPμ and to E-cadherin. Expression of PTPμ-extra (which lacks most of the cytoplasmic domain) induced adhesion to PTPμ but not to E-cadherin, demonstrating a requirement for the presence of the intracellular domains of PTPμ to restore E-cadherin-mediated adhesion. We previously observed a direct interaction between the intracellular domain of PTPμ and RACK1, a receptor for activated protein kinase C (PKC). We demonstrate that RACK1 binds to both the catalytically active and inactive mutant form of PTPμ. In addition, we determined that RACK1 binds to the PKCδ isoform in LNCaP cells. We tested whether PKC could be playing a role in the ability of PTPμ to restore E-cadherin-dependent adhesion. Activation of PKC reversed the adhesion of PTPμWT-expressing cells to E-cadherin, whereas treatment of parental LNCaP cells with a PKCδ-specific inhibitor induced adhesion to E-cadherin. Together, these studies suggest that PTPμ regulates the PKC pathway to restore E-cadherin-dependent adhesion via its interaction with RACK1.

A diverse set of cellular behaviors including growth, differentiation, adhesion, and migration are regulated by protein tyrosine phosphorylation. Protein tyrosine kinases and protein-tyrosine phosphatases (PTPs)[1] regulate intracellular phosphotyrosine levels. A subfamily of receptor-like PTPs (RPTPs) has extracellular segments containing adhesion molecule-like domains and intracellular segments that possess tyrosine phosphatase activity (1, 2). This structural arrangement suggests that RPTPs directly send signals in response to cell adhesion.

The receptor protein-tyrosine phosphatase PTPμ is a member of the Ig superfamily of adhesion molecules. The extracellular segment of PTPμ contains a MAM (Meprin/A5/PTPμ) domain, an Ig domain, and four fibronectin type III repeats (3). Expression of PTPμ induces aggregation of non-adherent cells (4, 5) through a homophilic binding site that resides within the Ig domain (6). The MAM domain plays a role in cell-cell aggregation by determining the specificity of the adhesive interaction (7). PTPμ contains a single membrane-spanning region with two cytoplasmic PTP domains. Only the membrane proximal PTP domain has catalytic activity (8). The role of the membrane distal PTP domain is not known, but this domain has been implicated in directing protein-protein interactions in other RPTPs (reviewed in Ref. 2). The intracellular juxtamembrane domain of PTPμ contains a region that is homologous to the conserved intracellular domain of the cadherins (9).

Cadherins are a family of calcium-dependent adhesion molecules that play an essential role in the formation of the cell-cell contacts termed adherens junctions (10). Cadherin-dependent adhesion is important for many physiological processes including establishment of cell polarity, morphogenetic movements such as epithelial/mesenchymal transitions, and cell type sorting during development (10, 11). Cadherins interact with the actin cytoskeleton via binding of the cytoplasmic domain to catenins (12). The catenins include α, β, γ/plakoglobin, and p120. β- and γ-catenin bind directly to the cytoplasmic segment of cadherin, whereas α-catenin binds to β- or γ-catenin thereby linking the cadherin-catenin complex to the cytoskeleton. Deletions in the catenin-binding region of cadherins disrupt cadherin-mediated adhesion despite the presence of an intact extracellular segment (12).

Despite the importance of cadherin-mediated cell-cell adhesion, the underlying mechanisms that regulate adhesion are still poorly understood. PTPμ has been shown to associate with the cadherin-catenin complex (13–15). Specifically, PTPμ interacts with a number of classical cadherins including E-cadherin, N-cadherin, and cadherin 4 (also called R-cadherin) (14). The classical cadherins have a highly conserved cytoplasmic domain, and PTPμ has been shown to bind directly to the C-terminal 38 amino acids of the intracellular domain of E-cadherin, which is the likely binding site in the other classical cadherins as well (14). We have shown that PTPμ regulates

* This work was supported in part by the Department of Defense Prostate Cancer Research Program Grant DAMD17-98-1-8586, managed by the United States Army Medical Research and Materiel Command, and a prostate pilot grant from the Case Western Reserve University Cancer Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; PKC, protein kinase C; WT, wild type; RPTP, receptor-like PTP; GFP, green fluorescent protein; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; Pipes, 1,4-piperazinediethanesulfonic acid; C-S, PTPμC1095S; PMA, phorbol 12-myristate 13-acetate.

N-cadherin-mediated neurite outgrowth (16). In fact, expression of a catalytically inactive form of PTPμ perturbed N-cadherin-mediated neurite outgrowth. This demonstrates that the phosphatase activity of PTPμ is required for N-cadherin-mediated signal transduction and/or regulation of the cytoskeleton in neurons.

In this study, we employed the LNCaP prostate carcinoma cell line (17) to investigate the role of PTPμ in E-cadherin-mediated adhesion. Unlike normal prostate epithelial cells, LNCaP cells do not express endogenous PTPμ. Although these cells express the proteins in the cadherin-catenin complex, we found that they did not mediate E-cadherin-dependent adhesion in aggregation assays and in an *in vitro* adhesion assay. By using a retroviral/tetracycline-repressible system, we re-expressed wild type and mutant forms of PTPμ in LNCaP cells and tested their effect on cell adhesion. Our data indicate that the cytoplasmic domain is important for restoring E-cadherin-dependent adhesion regardless of catalytic activity. In a recent paper (18), we isolated RACK1 as a PTPμ-interacting protein using a two-hybrid screen. RACK1 is a scaffolding protein that was originally identified as a receptor for activated protein kinase C (19). Because RACK1 binds activated protein kinase C, we tested whether PKC may play a role in the ability of PTPμ to regulate E-cadherin-mediated adhesion. Data presented here suggest that the cytoplasmic domain of PTPμ regulates E-cadherin-mediated adhesion through modulating PKC via the PTPμ/RACK1 interaction.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal antibodies against the intracellular (SK7) and extracellular (BK2) domains of PTPμ have been described (4, 6). A monoclonal antibody against γ-catenin (5172) was kindly provided by Dr. Pamela Cowin (New York University). Monoclonal antibodies against chick L1 (8D9) were generated in our lab using hybridoma cells generously provided by Dr. Vance Lemmon (Case Western Reserve University, Cleveland, OH). Monoclonal antibodies against E-cadherin, p120, α- and β-catenin, and RACK1 were purchased from Transduction Laboratories (Lexington, KY). A monoclonal antibody against vinculin and a monoclonal antibody against the extracellular domain of E-cadherin (DECMA) were purchased from Sigma. Goat anti-mouse IgG and IgM immunobeads were obtained from Zymed Laboratories Inc. Laboratories (San Francisco) or goat anti-mouse IgG immunobeads were alternatively obtained from Cappel (Costa Mesa, CA). Normal prostate epithelial cells were purchased from Clonetics (San Diego, CA). LY294002, PMA, rottlerin, chelerythrine chloride, GF109203X, and Gö6976 were purchased from Calbiochem. RPMI 1640 medium, SMEM medium, and laminin were obtained from Invitrogen. Fetal bovine serum was obtained from HyClone (Logan, UT). Tween 20 was obtained from Fisher. All other reagents were obtained from Sigma.

Construction and Expression of the PTPμ Retroviruses—The retroviral system used is a tetracycline-repressible ("tet-off") promoter-based system (20). By using the pBPSTR1 vector generously provided by Dr. Steven Reeves (Harvard Medical School, Charlestown, MA), the following constructs were generated: wild type PTPμ, the C-S mutant form of PTPμ, and PTPμ-extra. The wild type PTPμ plasmid (PTPμWT) and the PTPμC1095S (C-S) catalytically inactive mutant have been described previously (16). Briefly, the wild type PTPμ plasmid contained almost the entire coding sequence of PTPμ (base pairs 1–4350, *i.e.* it only lacked the last two amino acids and the stop codon). This was done to create an in-frame fusion with the green fluorescence protein at the C terminus (PTPμ-GFP). The mutant form of PTPμ is also GFP-tagged and contains a cysteine to serine mutation at residue 1095. A construct containing the extracellular, the transmembrane, and 55 amino acids of the intracellular domains has been previously described (4) (Note: PTPμ-extra is not GFP-tagged.) This construct was subcloned into the tetracycline-regulatable retroviral vector, pBPSTR1. Replication-defective amphotrophic retroviruses were made by transfecting the PA317 helper cell line (ATCC CRL-9078) with the respective PTPμ-containing plasmids. Control virus was generated by transfecting PA317 helper cells with the pBPSTR1 plasmid.

Expression and Purification of GST Fusion Proteins—An E-cadherin GST fusion protein construct containing amino acids 9–139 of mouse E-cadherin was obtained from Dr. Robert Brackenbury (University of Cincinnati, Cincinnati, OH). The E-cadherin GST fusion protein was constructed by restriction digest of pBATEM2 with *Pvu*II and *Hin*cII. The fragment was ligated into the *Sma*I site of pGEX-KG, which results in a fusion protein containing amino acids 9–139 of E-cadherin with GST at the N terminus. The GST fusion protein construct for expression of the entire extracellular domain of PTPμ has been described previously (4). Expression of GST-tagged proteins in *E. coli* was induced by isopropyl-1-thio-β-D-galactopyranoside. The bacteria were collected by centrifugation at 3000 × *g* for 10 min and lysed in PBS containing 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM benzamidine, sonicated, and centrifuged again at 3000 × *g* for 10 min to remove debris. The supernatant was passed over glutathione-Sepharose beads (Amersham Biosciences) and washed, and the bound protein was eluted with 10 mM glutathione as described previously (4).

Tissue Culture and Retroviral Infection of LNCaP Cells—LNCaP cells (17) were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1 μg/ml gentamicin at 37 °C and 5% CO_2. Cells were infected with retrovirus by the addition of Polybrene (5 μg/ml) and virus-containing medium. The cells were incubated overnight at 37 °C, and the medium was exchanged with normal culture medium. Five days after infection, the cells were checked for expression of the PTPμ proteins, which were tagged with the green fluorescent protein (GFP) by fluorescence microscopy.

Protein Extraction and Immunoblotting—LNCaP cells were rinsed once with PBS, and the cells were lysed in Triton-containing buffer (20 mM Tris, pH 7.6, 1% Triton X-100, 2 mM $CaCl_2$, 1 mM benzamidine, 200 μM phenylarsine oxide, 1 mM vanadate, 0.1 mM ammonium molybdate, and 2 μl/ml protease inhibitor mixture) and scraped off the dish. In all experiments where RACK1 was co-immunoprecipitated, the cells were lysed in a buffer containing 20 mM Tris, pH 7.6, 1% Triton X-100, 50 mM NaCl, 1 mM benzamidine, 1 mM vanadate, and 2 μl/ml protease inhibitor mixture. After incubation on ice for 30 min, the lysate was centrifuged at 14,000 rpm for 3 min, and the Triton-soluble material was recovered in the supernatant. The amount of protein was determined by the Bradford method using BSA as a standard. Lysates were boiled in equal volume of 2× sample buffer, and the proteins were separated by 6 or 10% SDS-PAGE and transferred to nitrocellulose for immunoblotting as described previously (4).

Immunoprecipitations—Antibodies (5 μg of IgG/IP or 1.25 μg of IgM/IP) were incubated with goat anti-mouse IgG or IgM immunobeads, respectively, for 2 h at room temperature and then washed 3 times with PBS (9.5 mM phosphate, 137 mM NaCl, pH 7.5). Immunoprecipitates were prepared by incubating lysates containing either 250 μg of protein (Fig. 5) or 400 μg of protein (Fig. 6) with antibody-coupled beads overnight at 4 °C. The beads were washed extensively with lysis buffer, then boiled in sample buffer, and separated by 6 or 10% SDS-PAGE. One-fifth of the immunoprecipitate was loaded per lane. Proteins were transferred to a nitrocellulose membrane and immunoblotted as described (4).

Calcium-dependent Aggregation Assay—Aggregation assays were performed as described previously (21). Briefly, cells were trypsinized in the presence of calcium, which selectively preserved cadherins (22). Uninfected LNCaP cells or cells infected with either PTPμWT or C-S mutant were rinsed twice in HCMF buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 5.4 mM KCl, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 5.5 mM glucose) containing 2 mM $CaCl_2$ and trypsinized into single cells by incubation with 0.04% trypsin in HMCF buffer supplemented with 2 mM $CaCl_2$. The trypsin was inactivated by the addition of RPMI containing 10% serum. The cells were pelleted and resuspended in 2 ml of SMEM, followed by a 20-min incubation with 20 units/ml DNase on ice. Some of the cells re-expressing PTPμWT were treated with 200 μg/ml of an E-cadherin function-blocking antibody (DECMA) for an additional 20 min on ice. 2×10^6 cells were added to scintillation vials containing HMCF buffer with a final concentration of 2 mM $CaCl_2$. Where indicated, the $CaCl_2$ was substituted with 5 mM EDTA (final concentration). Aggregation was initiated by placing the vials at 37 °C at 90 rpm in a gyratory shaker. Aliquots of the samples were diluted 50-fold in PBS, and the number of particles were determined using a Coulter Counter. The Coulter Counter was set at a lower threshold of 10%, 1/aperture current of 16, 1/amplification of 2. Percent aggregation was calculated by subtracting the number of particles after 1 h (N_t) from the initial particle number and dividing by the initial number $\{((N_0 - N_t)/N_0) \times 100\}$.

LNCaP Adhesion to Purified Proteins—Sterile coverslips were coated overnight with 100 μg/ml poly-L-lysine (Sigma), washed twice in sterile water, and allowed to dry. Subsequently, the coverslips were coated with nitrocellulose in methanol (23) and allowed to dry. Purified recombinant proteins were diluted in PBS containing 2 mM $CaCl_2$ to a con-

centration of 75 μg/ml for PTPμ and E-cadherin, respectively, and 40 μg/ml for laminin. To identify the individual protein spots on the coverslips, the protein solutions were supplemented with 20 μg/ml Texas Red BSA (Sigma). Three distinct spots, each containing a single adhesion molecule (laminin, E-cadherin, and PTPμ), were generated by spotting 20 μl of each protein solution on one coverslip. After a 20-min incubation at room temperature, the protein solutions were aspirated, and this procedure was repeated once. Remaining binding sites on the nitrocellulose were blocked with 2% BSA in PBS, and the dishes were rinsed with RPMI 1640 medium. LNCaP cells infected with the indicated retrovirus were fully trypsinized with 0.05% trypsin, 0.53 mM EDTA (Invitrogen), and 3×10^5 cells were added to coverslips, and the cells were allowed to adhere overnight to regenerate cell surface proteins. In the control experiments, function-blocking antibodies to either PTPμ (BK2, 10 μl/ml ascites) or E-cadherin (DECMA, 1:1 dilution of culture supernatant), or 5 mM EDTA (final concentration) was added to the dishes just prior to the addition of cells. In some experiments, the overnight incubation was followed by a 15-min incubation with 20 nM PMA or an equal volume of Me_2SO. Alternatively, uninfected LNCaP cells were added to coverslips and incubated overnight followed by a 45-min incubation with either 5 μM rottlerin, 10 μM chelerythrine chloride, 0.5 μM GF109203X, 15 nM Gö6976, 10 μM LY294002, or Me_2SO alone. At the concentrations used, chelerythrine chloride (IC_{50} = 0.66 μM) and GF109203X (IC_{50} ranges between 8 nM and 5.8 μM for different isoforms of PKC) are specific for PKC, whereas rottlerin is specific for PKCδ (IC_{50} = 6 μM), and Gö6976 is specific for PKCα and -β (IC_{50} = 2.3 and 6 nM, respectively). LY294002 inhibits the phosphatidylinositol 3-kinase (IC_{50} = 1.4 μM). The medium was then removed, and the coverslips were rinsed once in PBS to remove unattached cells. The cells were subsequently fixed with 4% paraformaldehyde, 0.01% glutaraldehyde in PEM buffer (80 mM Pipes, 5 mM EGTA, 1 mM $MgCl_2$, 3% sucrose), pH 7.4, for 30 min at room temperature. The coverslips were washed twice in PBS and mounted in IFF mounting medium (0.5 M Tris-HCl, pH 8.0, containing 20% glycerol, and 0.1% *p*-phenylenediamine). Adherent cells were detected by dark field microscopy, using a 5× objective, and photographed. To quantify the number of adherent cells, the 35-mm negatives were scanned, and the digitized images were analyzed using the Metamorph image analysis program (Universal Imaging Corp., West Chester, PA). The number of adherent cells per image was approximated by highlighting the cells using the threshold function, and the total number of highlighted cells per image was calculated. The data obtained in 4–6 separate experiments were analyzed by Student's *t* test (Statview 4.51, Abacus Concepts, Inc.).

RESULTS

Re-expression of PTPμ—The receptor tyrosine phosphatase PTPμ has been shown previously to interact with E-cadherin in a variety of tissues by immunoprecipitation (13–15). To investigate whether PTPμ plays a functional role in E-cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cell line (17). Unlike normal prostate epithelial cells (NPr), these cells do not express PTPμ (Fig. 1*a*, *VEC*). To re-express PTPμ in LNCaP cells, we generated a tetracycline-regulatable retrovirus encoding the PTPμ cDNA sequence tagged with the green fluorescence protein (PTPμ-GFP) (16). By using this retrovirus, we re-expressed wild type PTPμ (PTPμWT) in the LNCaP cells. Five days after retroviral infection, the cells were analyzed for expression of PTPμWT-GFP by immunoblot and by fluorescence microscopy. Immunoblot analysis showed that LNCaP cells infected with retrovirus containing an empty vector do not express PTPμ (Fig. 1*a*, *VEC*). Cells infected with retrovirus containing PTPμWT (Fig. 1*a*, *WT*) expressed both the full-length protein (200 kDa) as well as the proteolytically processed forms (~100 kDa) (6). Due to the GFP tag, both the full-length and the proteolytically processed forms of the re-expressed PTPμWT migrated at a higher molecular weight than the PTPμ expressed in normal prostate cells (Fig. 1*a*, *NPr*). The retroviral system we used is a tet-off system, and in the presence of tetracycline the gene is not expressed. The re-expression of PTPμWT was inhibited by treating the cells with tetracycline (Fig. 1*a*, *WT+T*). Fluorescence microscopy revealed that between 70 and 90% of the LNCaP cells expressed PTPμWT and that PTPμ was primarily localized to the plasma membrane as expected (Fig. 2, *C* and *D*). This expression was repressed when the cells were grown in the presence of 4 μg/ml tetracycline (Fig. 2, *E* and *F*). Control cells infected with a virus containing an empty vector did not show any fluorescence (Fig. 2, *A* and *B*).

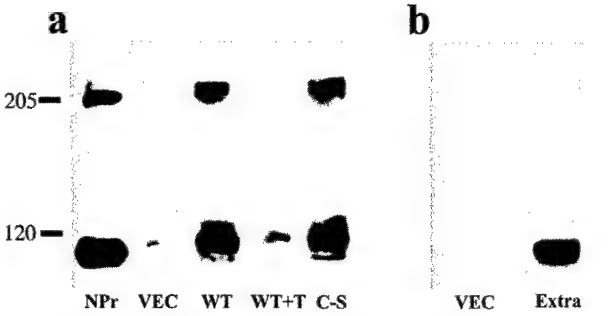

FIG. 1. **Re-expression of PTPμ in LNCaP cells.** LNCaP cells were infected with retrovirus containing an empty vector (*VEC*), PTPμWT (*WT*), a mutant form of PTPμ containing a single point mutation in the catalytic site (*C-S*), or a construct containing the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (*Extra*). To verify that protein expression is under tetracycline control, 4 μg/ml tetracycline was added daily to cells infected with retrovirus containing PTPμWT (*WT+T*). Five days after infection, the cells were lysed, and 30 μg of lysate from normal prostate epithelial cells (*NPr*) and LNCaP cells were separated by 6% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with monoclonal antibodies to PTPμ. Note that both PTPμWT and the C-S mutant have a GFP tag and therefore migrate at a higher molecular weight than PTPμ expressed in the normal prostate epithelial cells. *a*, Western blot using the monoclonal antibody SK7 against the intracellular domain of PTPμ. *b*, Western blot using the monoclonal antibody BK2 against the extracellular domain of PTPμ.

To assess the functional role of PTPμ catalytic activity in the regulation of E-cadherin-mediated adhesion, we have generated tetracycline-repressible retrovirus encoding a mutant form of PTPμ-GFP containing a single amino acid mutation in the catalytic site (16). Mutation of the conserved cysteine residue PTPμC1095S (C-S) results in a catalytically inactive enzyme. Immunoblot analysis showed that the C-S mutant was expressed at a similar level to PTPμWT in LNCaP cells (Fig. 1*a*, *C-S*). Fluorescence microscopy confirmed that infection with the C-S mutant (Fig. 2, *G* and *H*) resulted in expression at the plasma membrane at a similar level as PTPμWT, demonstrating that the expression and subcellular localization are not affected by the loss of catalytic activity.

Re-expression of PTPμ Enhanced Calcium-dependent Aggregation of LNCaP Cells—To investigate whether PTPμ plays a role in E-cadherin-mediated adhesion in LNCaP cells, we trypsinized the cells in the presence of $CaCl_2$ to selectively preserve the cadherins (22). This assay only measures calcium-dependent aggregation predominantly mediated by the cadherins (22). The cells were then allowed to aggregate for 1 h. LNCaP cells infected with an empty vector weakly aggregated (27.4%). Re-expression of PTPμWT increased the aggregation 3-fold (72.4%), as did expression of the C-S mutant (72.5%). The increased aggregation was only partly dependent on E-cadherin function, because the presence of an E-cadherin function-blocking antibody did not completely reduce the aggregation induced by re-expression of PTPμ (49.7%). However, the increased aggregation was Ca^{2+}-dependent, because the presence of EDTA reduced the aggregation to a level below that seen in cells infected with an empty vector (10.8%). The residual Ca^{2+}-dependent adhesion is at least due in part to the fact that LNCaP cells express N-cadherin (data not shown). Taken together, these findings demonstrate that re-expression of PTPμ in LNCaP cells induced Ca^{2+}-dependent aggregation that is partly because of E-cadherin-dependent cell-cell adhe-

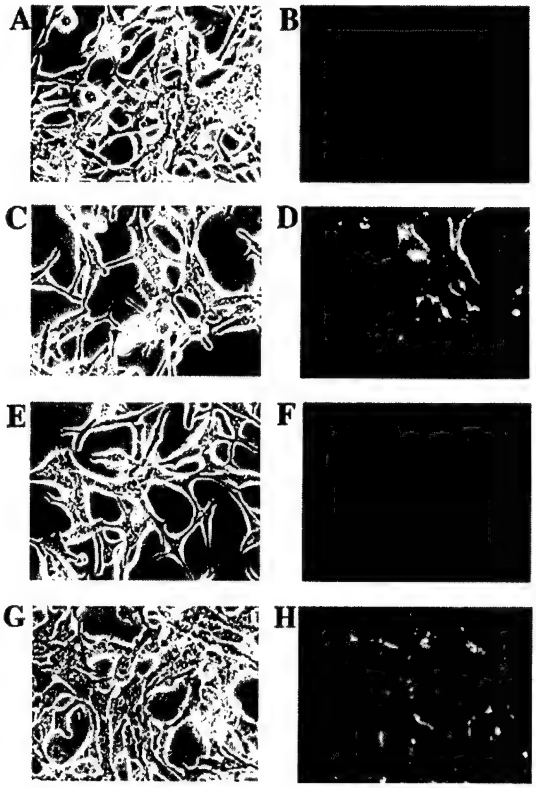

FIG. 2. **GFP-tagged forms of PTPμ are expressed at the cell surface and regulated by tetracycline.** LNCaP cells were infected with a retrovirus containing an empty vector (*A* and *B*), wild type PTPμ tagged with GFP in the absence (*C* and *D*) or presence (*E* and *F*) of 4 μg/ml tetracycline, or with a mutant form of PTPμ containing a single C-S point mutation in the catalytic site (*G* and *H*). Five days after infection, the expression of GFP-tagged proteins was visualized by fluorescence microscopy (×128 magnification). Representative phase contrast (*A, C, E,* and *G*) and fluorescent (*B, D, F,* and *H*) images are shown.

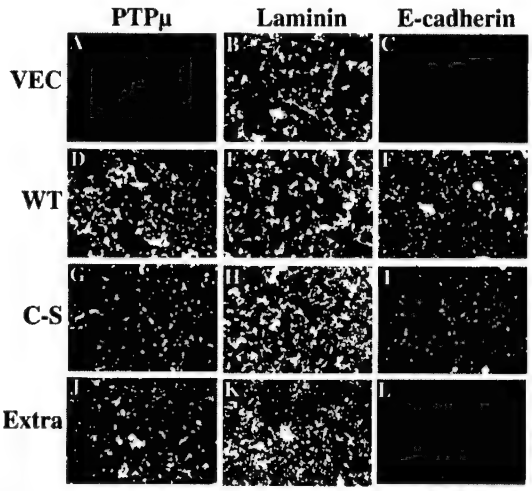

FIG. 3. **LNCaP adhesion to purified recombinant proteins.** LNCaP cells were infected with retrovirus containing an empty vector (*A–C*), PTPμWT (*D–F*), a catalytically inactive mutant form of PTPμ(C-S) (*G–I*), or PTPμ-extra (*J–L*). Five days after infection, the cells were incubated with coverslips spotted with purified recombinant PTPμ (*A, D, G,* and *J*), laminin (*B, E, H,* and *K*), or E-cadherin (*C, F, I,* and *L*). Virtually the entire protein spot is visible in the field shown in each panel. Adherent cells were visualized by darkfield microscopy (×16 magnification) and photographed using a 35-mm camera.

sion. Thus, aggregation assays were not ideal to specifically study E-cadherin-dependent adhesion in LNCaP cells. Therefore, we utilized an *in vitro* adhesion assay that measures specific binding to a given adhesion molecule which is similar to our previously published assay (4).

Re-expression of PTPμ-induced Adhesion to Purified PTPμ—To study the specific interactions between cell-cell adhesion molecules in LNCaP cells, we developed an *in vitro* adhesion assay where purified, recombinant proteins were immobilized on nitrocellulose-coated coverslips. Basically, three spots of protein (laminin, E-cadherin, and PTPμ) were added to each nitrocellulose-coated coverslip. The field shown in each panel represents virtually the entire spot for a given adhesion molecule. PTPμ has been shown to mediate cell-cell adhesion via homophilic binding (4, 5). To verify that the re-expressed forms of PTPμ were able to mediate homophilic binding in LNCaP cells, we investigated the adhesion of LNCaP cells to purified recombinant PTPμ that was immobilized on nitrocellulose-coated coverslips. As expected, cells infected with an empty vector did not adhere to PTPμ (Fig. 3*A*) because these cells do not express PTPμ. Re-expression of PTPμWT induced LNCaP adhesion to purified PTPμ (Fig. 3*D*), as did re-expression of the C-S mutant (Fig. 3*G*). Quantitation of the adhesion assays ($n = 6$) showed that the number of cells that adhered to purified PTPμ was significantly higher for cells infected with both the WT and the C-S mutant form of PTPμ as compared with cells infected with vector only (Table I). However, there was no difference between cells expressing PTPμWT compared with the C-S mutant in their ability to adhere to purified PTPμ (Table I). To ensure the specificity of the adhesion assay, we repeated the experiments in the presence of function-blocking antibodies to either PTPμ or E-cadherin. The presence of an antibody to the extracellular domains of PTPμ specifically inhibited the adhesion to recombinant PTPμ of LNCaP cells re-expressing PTPμWT (Fig. 4*a*, *WT+PTPμ Ab*) or cells expressing the C-S mutant (Fig. 4*a*, *C-S+PTPμ Ab*). As expected, the presence of the E-cadherin antibody had no effect on adhesion to PTPμ (Fig. 4*a*, *WT+E-ca. Ab, C-S+E-cad Ab*). Taken together, these data confirm that the re-expressed PTPμ is present at the cell surface and capable of mediating homophilic binding. In addition, PTPμ phosphatase activity is not necessary for PTPμ-dependent adhesion to occur as demonstrated previously (4).

As an internal control in each experiment, cells were allowed to adhere to laminin. Adhesion to extracellular matrix proteins such as laminin is mediated through integrin receptors. Because there is no evidence indicating that PTPμ regulates integrin function, LNCaP adhesion to laminin should not be affected by the re-expression of PTPμ. As expected, LNCaP cells infected with an empty vector adhered to laminin (Fig. 3*B*), and this adhesion was not significantly affected by re-expression of either WT (Fig. 3*E*) or C-S mutant forms of PTPμ (Fig. 3*H* and Table I). None of the retrovirally infected cells adhered to nitrocellulose coated with BSA only (data not shown).

Re-expression of PTPμ Restores E-cadherin-mediated Adhesion—To study the role of PTPμ in the regulation of E-cadherin-mediated adhesion in LNCaP cells, we immobilized purified recombinant E-cadherin on the nitrocellulose-coated coverslips. Despite the fact that these cells express E-cadherin as well as α-, β-, and γ- catenin and p120 (Fig. 5), LNCaP cells infected with an empty vector did not adhere to E-cadherin (Fig. 3*C*). Re-expression of PTPμWT restored the ability of LNCaP cells to adhere to E-cadherin (Fig. 3*F*). Quantitation of the adhesion assays show that the number of cells infected with PTPμWT that adhered to E-cadherin was significantly higher than the number of cells infected with vector only (Table I). These data show that expression of PTPμ is necessary for E-cadherin-mediated adhesion in LNCaP cells.

TABLE I

Statistical analysis of LNCaP adhesion to purified recombinant proteins

The data shown in Figs. 4 and 7*a* are presented as mean number of adherent cells, (−S.E.) Six independent adhesion assays (from six different experiments) were averaged to produce the mean number of adherent cells. For each substrate, Student's *t* test was used to compare the number of cells infected with PTPμ with the number of adherent cells infected with an empty vector.

Virus type	Substrate					
	PTP		Laminin		E-cadherin	
	Adherent cells	p^a	Adherent cells	p^a	Adherent cells	p
Vector	95.5–45.7		1483.4–368.2		132.7–62.8	
PTP WTGFP	1150.0–210.8	0.0006	1391.8–192.7	0.8311	1561.5–350.0	0.0024
C1095S	1022.5–192.0	0.0008	1736.2–463.0	0.6804	1117.8–210.9	0.0012
PTP-extra	1102.0–240.1	0.0021	2432.4–431.3	0.1328	48.0–20.0	0.2281
Vector	45.2–6.82		1335.2–573.9		34.8–14.0	
Vector + PMA	54.8–11.5	0.5039	1799.2–543.3	0.5733	36.0–6.2	0.8985
PTP WT	1082.8–189.3	0.0015	1346.6–206.1	0.9855	1047.8–189.5	0.0003
PTP WT + PMA	1521.3–349.4	0.0055	1442.8–257.1	0.8684	120.2–50.5	0.1312

[a] *p* values were obtained by Student's *t* test, 99% confidence interval.

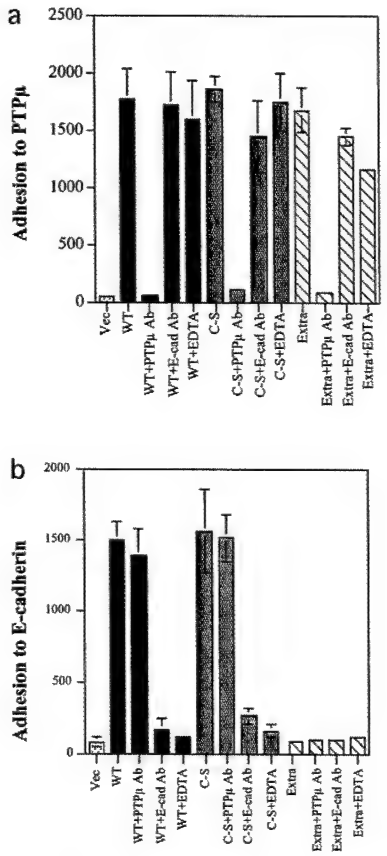

FIG. 4. **Specificity of the adhesion assay.** LNCaP cells were infected with retrovirus containing an empty vector (*VEC*), PTPμWT (*WT*), a catalytically inactive mutant form of PTPμ (*C-S*), or PTPμ-extra (*Extra*). Five days after infection, the cells were incubated with coverslips spotted with purified recombinant PTPμ (*A*) or E-cadherin (*B*) in the presence of either a PTPμ antibody (*BK2*), an E-cadherin antibody (DECMA), 5 mM EDTA, or were left untreated. Adherent cells were fixed after an overnight incubation and were visualized by darkfield microscopy and photographed using a 35-mm camera. The 35-mm negatives from six experiments were scanned, and the digitized images were analyzed using the Metamorph image analysis program. To measure the number of adherent cells per image, the cells were highlighted using the threshold function, and the total number of highlighted cells per image was calculated. The data is presented as mean ± S.E.

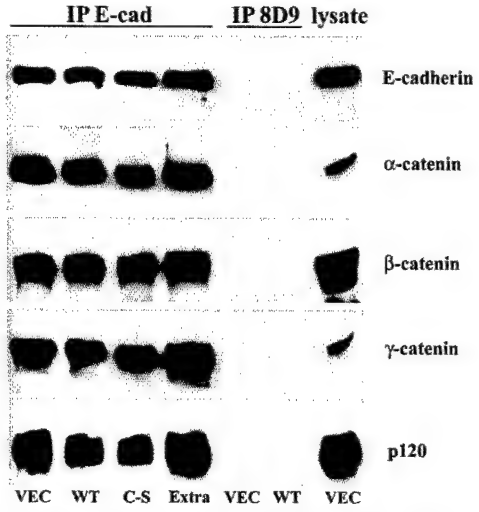

FIG. 5. **Immunoprecipitation of E-cadherin.** LNCaP cells were infected with retrovirus containing an empty vector (*VEC*), PTPμWT (*WT*), a catalytically inactive mutant form of PTPμ (*C-S*), or PTPμ-extra (*Extra*). Five days after infection, the cells were lysed, and 250 μg of the lysates were subjected to immunoprecipitation (*IP*) using antibodies to E-cadherin (*E-cad*) or L1 (*8D9*). The immunoprecipitates were separated by 6% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to the indicated proteins. As a positive control, 30 μg of lysate from vector-infected cells (*lysate*) is shown in each panel.

Because tyrosine phosphorylation has been reported to negatively regulate cadherin-mediated adhesion, we investigated whether PTPμ restored E-cadherin-mediated adhesion by dephosphorylating key components of the cadherin-catenin complex. To do this, we repeated the adhesion assays with cells expressing the C-S mutant form of PTPμ. Expression of the C-S mutant restored E-cadherin-mediated adhesion (Fig. 3*I*). As shown in Table I, re-expression of WT or the C-S mutant form of PTPμ induced a significant increase in adhesion to E-cadherin as compared with LNCaP cells infected with an empty vector. In contrast, there was no difference in adhesion between cells infected with PTPμWT compared with cells infected with the C-S mutant. The statistical analysis for LNCaP cell adhesion to E-cadherin is summarized in Table I. In control experiments, the adhesion to E-cadherin was totally blocked by a function-blocking antibody to E-cadherin (Fig. 4*b*, *WT+E-cad Ab* and *C-S+E-cad Ab*, respectively). In contrast, the PTPμ antibody did not affect the adhesion to E-cadherin induced by the re-expression of PTPμWT (Fig. 4*b*, *WT+PTPμ Ab*) or by the expression of the C-S mutant (Fig. 4*b*, *C-S+PTPμ Ab*) as expected. E-cadherin-mediated adhesion in this assay is Ca^{2+}-dependent, and addition of 5 mM EDTA abolished adhesion to E-cadherin (Fig. 4*b*, *WT+EDTA* and *C-S+EDTA*, respectively). However, the presence of EDTA did not affect the adhesion to PTPμ, which is calcium-independent (4), of cells either re-expressing PTPμ-WT (Fig. 4*a*, *WT+EDTA*) or expressing the C-S mutant (Fig. 4*a*, *C-S+EDTA*). Taken together, these data

indicate that although the presence of the PTPμ protein is required for E-cadherin-mediated adhesion in LNCaP cells, it does not require PTPμ catalytic activity.

It is possible that the PTPμ intracellular domain may recruit other proteins that aid in restoring E-cadherin-mediated adhesion. To determine whether the intracellular PTP domains of PTPμ were required to affect E-cadherin-dependent adhesion, we constructed a retrovirus encoding the extracellular, transmembrane, and 55 amino acids of the intracellular domains of PTPμ (PTPμ-extra) (4). Western blot analysis confirmed that this construct was expressed in LNCaP cells (Fig. 1*b*, *Extra*). The cytoplasmic domain of PTPμ is known to bind to E-cadherin (13). Immunoprecipitation experiments confirmed that PTPμ-extra does not associate with E-cadherin (data not shown). Expression of PTPμ-extra induced LNCaP adhesion to purified recombinant PTPμ (Fig. 3*J*; Table I), confirming that the intracellular domains are not required for PTPμ to mediate homophilic binding (4). In addition, adhesion to PTPμ was blocked by an antibody to PTPμ (Fig. 4*a*, *Extra+PTPμ Ab*). The antibody to E-cadherin and 5 mM EDTA had no major effect on the adhesion to PTPμ (Fig. 4*a*, *Extra+E-cad Ab* and *Extra+EDTA*, respectively) as expected. However, PTPμ-extra did not restore LNCaP adhesion to recombinant E-cadherin (Fig. 3*L*), demonstrating that the intracellular domains of PTPμ are necessary for restoring E-cadherin-mediated adhesion. Because LNCaP cells expressing PTPμ-extra did not adhere to E-cadherin (Fig. 3*L*; Fig. 4*b*, *Extra*), the presence of either the PTPμ antibody, the E-cadherin antibody, or 5 mM EDTA had no effect on adhesion to E-cadherin (Fig. 4*b*, *Extra + PTPμ Ab*, *Extra + E-cad Ab*, and *Extra + EDTA*, respectively). As expected, the expression of PTPμ extra did not affect LNCaP adhesion to laminin (Fig. 3*K*). Together, these results suggest that PTPμ-extra is expressed at the cell surface and capable of inducing adhesion to PTPμ but not restoring E-cadherin-dependent adhesion.

Similar to the results shown in Fig. 3, LNCaP cells expressing an empty vector did not adhere to either PTPμ or E-cadherin (Fig. 4*a*, *VEC*, and Fig. 4*b*, *VEC*, respectively), and this was not altered by the presence of either the PTPμ antibody, the E-cadherin antibody, or 5 mM EDTA (data not shown). Taken together, these experiments demonstrate that this *in vitro* adhesion assay can be used to study specific binding to cell-cell adhesion molecules.

Expression of Cadherins and Catenins—Cadherin-mediated cell-cell adhesion is dependent on the expression of both cadherins and catenins. Immunoblot analysis demonstrated that LNCaP cells expressed E-cadherin as well as α-, β-, and γ-catenin and p120 (Fig. 5, *lysate*). This is in accordance with normal prostate epithelial cells, which were found to express similar amounts of E-cadherin as well as α-, β-, and γ-catenin (data not shown). Infection of LNCaP cells with an empty vector, PTPμWT, or the C-S mutant form of PTPμ did not alter the expression of any of the proteins in the cadherin-catenin complex (Fig. 5). It is possible that re-expression of wild type or mutant forms of PTPμ may alter the subcellular localization of the proteins in the cadherin-catenin complex, thereby altering the function of the complex. To address this question, we performed immunocytochemical analysis on LNCaP cells using antibodies to E-cadherin as well as α-, β-, and γ-catenin and p120. However, re-expression of either PTPμWT or the C-S mutant did not significantly alter the subcellular localization of any of the proteins examined (data not shown).

PTPμ Does Not Alter the Association of α-, β-, γ-Catenin or p120 to E-cadherin—To examine the possibility that the presence of PTPμ affects the binding of α-, β-, and γ-catenin or p120 to E-cadherin, we immunoprecipitated E-cadherin from cells

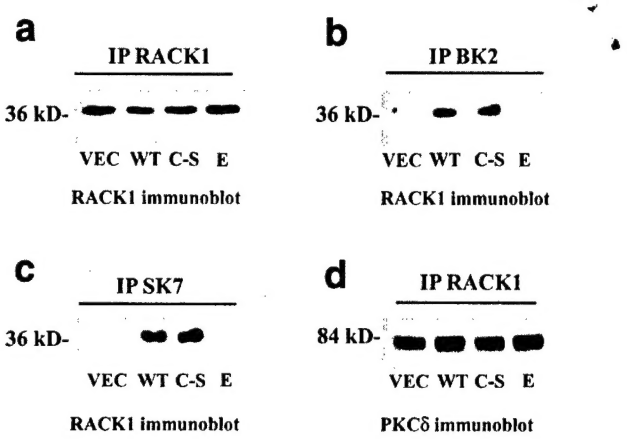

FIG. 6. **PTPμ interacts with RACK1 regardless of catalytic activity.** LNCaP cells were infected with retrovirus containing an empty vector (*VEC*), PTPμWT (*WT*), a catalytically inactive mutant form of PTPμ (*C-S*), or PTPμ-extra (*E*). Five days after infection, the cells were lysed, and 400 μg of the lysates were subjected to immunoprecipitation using a monoclonal antibody generated against RACK1 (*a*), a monoclonal antibody generated against the extracellular domain of PTPμ (*BK2*) (*b*), or a monoclonal antibody generated against the intracellular domain of PTPμ (*SK7*) (*c*). The immunoprecipitates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies generated against RACK1 (*a–c*). *d*, the immunoblot of RACK1 immunoprecipitates shown in *a* was stripped and reprobed with a polyclonal antibody generated against PKCδ.

infected with an empty vector (*VEC*), PTPμWT (*WT*), C-S, or PTPμ-extra (*Extra*). As shown in Fig. 5, the immunoprecipitates from cells infected with an empty vector, PTPμWT, as well as the C-S and PTPμ-extra contained equal amounts of E-cadherin. The immunoblot was stripped and reprobed with antibodies to the catenins. Immunoprecipitates from cells infected with an empty vector contained α-, β-, and γ-catenin as well as p120. Infection of cells with various forms of PTPμ did not significantly alter the amounts of the catenins that co-immunoprecipitated with E-cadherin. As a control, a monoclonal antibody to chick L1 (8D9) was used. This antibody did not immunoprecipitate either E-cadherin or any of the catenins.

The PTPμ Cytoplasmic Domain, Regardless of Catalytic Activity, Is Required for the Interaction with RACK1—Even though the presence of PTPμ does not alter the composition of the E-cadherin-catenin complex, it is possible that full-length PTPμ regulates E-cadherin-dependent adhesion by recruiting other signaling molecules to the cadherin-catenin complex. In a recent paper (18), we demonstrated an interaction between the membrane-proximal phosphatase domain of PTPμ and RACK1, a receptor for activated PKC (19). Because RACK1 binds to the catalytic domain of PTPμ, we tested whether catalytic activity of PTPμ was required to interact with RACK1. We performed immunoprecipitation with antibodies directed against PTPμ or RACK1 and subjected the immunoprecipitates to SDS-PAGE and immunoblotted the gels with anti-RACK1 antibodies. Immunoprecipitation of RACK1 showed that LNCaP cells infected with an empty vector expressed RACK1 (Fig. 6*a*, *VEC*) and that infection of cells with various forms of PTPμ did not alter the expression of RACK1 (Fig. 6*a*, *WT*, *C-S*, and *E*, respectively). To investigate whether PTPμWT and the C-S mutant associate with RACK1 in LNCaP cells, we immunoprecipitated PTPμ using an antibody to the extracellular domain of PTPμ (BK2). RACK1 was found to associate with both PTPμWT (Fig. 6*b*, *WT*) and the C-S mutant (Fig. 6*b*, *C-S*) but not with PTPμ-extra (Fig. 6, *b*, *E*). As expected, PTPμ antibody did not immunoprecipitate RACK1 from cells infected with an empty vector (Fig. 6*b*, *VEC*). This experiment was repeated with an antibody to the intracellular do-

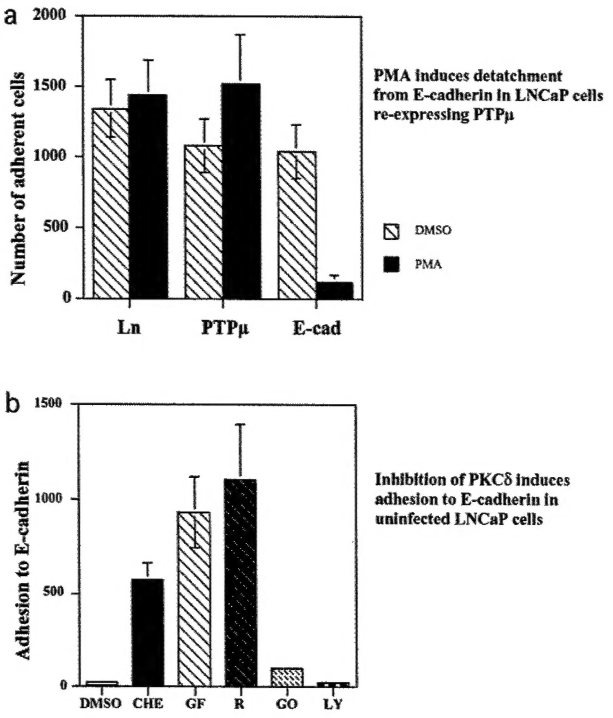

FIG. 7. **Protein kinase C regulates LNCaP adhesion to E-cadherin.** *a*, PMA induced the detachment of cells re-expressing PTPμWT from E-cadherin but not from PTPμ or laminin. LNCaP cells were infected with retrovirus containing PTPμWT. Five days after adhesion, the cells were added to coverslips spotted with purified recombinant PTPμ, laminin, or E-cadherin. After an overnight incubation, the cells were treated with Me_2SO or 20 nM PMA for 15 min. *b*, inhibition of PKCδ induced adhesion to E-cadherin in uninfected LNCaP cells. Uninfected LNCaP cells were incubated overnight with coverslips spotted with purified recombinant E-cadherin, followed by a 45-min incubation with 10 μM chelerythrine chloride (*CHE*), 0.5 μM GF109203X (*GF*), 5 μM rottlerin (*R*), 15 nM Gö6976 (*Go*), 10 μM LY294002 (*LY*), or with Me_2SO alone. Adherent cells were fixed and visualized by darkfield microscopy and analyzed using the Metamorph image analysis program. The data are presented as mean ± S.E.

main of PTPμ (SK7). As seen in Fig. 6*c*, the SK7 antibody also co-immunoprecipitated RACK1 from cells infected with PTPμWT or C-S but not from cells infected with PTPμ-extra or an empty vector. Taken together, these data demonstrate that full-length PTPμ regardless of its catalytic activity associates with RACK1.

The association between PTPμ and RACK1 suggests that the presence of the PTPμ protein in LNCaP cells may regulate the PKC pathway, which could be involved in the restoration of E-cadherin-mediated adhesion by PTPμ. Several studies (24–26) have shown that activation of the PKC pathway can either up-regulate or down-regulate E-cadherin-mediated adhesion depending on cell type. Therefore, we investigated whether activation of PKC by PMA affects the ability of PTPμ to restore E-cadherin-mediated adhesion. LNCaP cells infected with retrovirus containing PTPμWT were allowed to adhere to PTPμ, laminin, or E-cadherin as described above. The cells were then treated with PMA for 15 min to activate PKC, which did not affect adhesion to either PTPμ or laminin (Fig. 7*a*). However, activation of PKC detached the PTPμ-expressing cells from E-cadherin (Fig. 7*a*). The statistical analyses for the effects of PMA on LNCaP adhesion are shown in Table I.

Inhibition of PKCδ Induced LNCaP Adhesion to E-cadherin—To examine further the signal transduction pathways involved in restoring E-cadherin-mediated adhesion, we studied the effect of various kinase inhibitors on the ability of LNCaP cells to adhere to E-cadherin. Uninfected LNCaP cells were added to coverslips with immobilized E-cadherin, and the cells were subsequently treated with the inhibitors. Chelerythrine chloride and GF109203X are broad specificity compounds that inhibit most PKC isoforms. Rottlerin only inhibits the PKCδ isoform of PKCs. We found that treatment of the cells with three different PKC inhibitors, chelerythrine chloride, GF109203X, and a PKCδ-specific inhibitor (rottlerin) induced LNCaP adhesion to E-cadherin (Fig. 7*b*, *CHE*, *GF*, and *R*, respectively). The induction of E-cadherin-dependent adhesion was due to inhibition of PKCs in general but we believe specific for inhibition of PKCδ, because treatment of LNCaP cells with the PKCα- and PKCβ-specific inhibitor Gö6976 did not induce adhesion (Fig. 7*b*, *Go*). In addition, neither Me_2SO nor the phosphatidylinositol 3-kinase inhibitor LY294002 induced LNCaP adhesion to E-cadherin (Fig. 7*b*, *Me_2SO* and *LY*, respectively). Also, the effect of the PKCδ inhibition was specific in that it only affected the ability of LNCaP cells to adhere to E-cadherin but did not alter adhesion to laminin (data not shown). Interestingly, the PMA-induced detachment of the PTPμ-expressing cells from E-cadherin was blocked by preincubating the cells with rottlerin (data not shown). Furthermore, PKCδ was found to associate with RACK1 in LNCaP cells, although this association was not affected by the presence of either PTPμWT or the C-S mutant (Fig. 6*d*) (19). This result is not surprising because our experiments were done in the presence of serum, which activates PKC. Our data suggest that PTPμ negatively regulates PKCδ activity to restore E-cadherin-dependent adhesion. However, the precise mechanism of PKCδ regulation by PTPμ is not clear but is likely to involve RACK1. Taken together, these data indicate that PTPμ may restore E-cadherin-mediated adhesion in LNCaP cells by regulating the PKC pathway through the recruitment of RACK1 to the PTPμ complex.

DISCUSSION

Alterations in the function of the E-cadherin/catenin adhesion system occur frequently in a wide variety of human carcinomas (27). The molecular mechanisms underlying the loss of expression or functionality of individual components of the cadherin-catenin complex is still only partly understood. Previous studies (13–15) have shown that PTPμ associates with classical cadherins. The functional importance of this interaction was illustrated in a study from our lab where we demonstrated that PTPμ regulates N-cadherin-mediated neurite outgrowth of retinal ganglion cells (16). Therefore, it is possible that a loss of expression or function of PTPμ may result in a defect in cadherin-mediated adhesion. To investigate the role of PTPμ in E-cadherin-mediated adhesion, we employed the LNCaP prostate carcinoma cell line (17). These cells provide a good model system in that they, unlike normal prostate epithelial cells, do not express endogenous PTPμ. This allowed us to re-express PTPμ and study the effects of wild type (WT) as well as mutant forms of PTPμ without the interference of endogenous PTPμ. Retroviral re-expression of both WT and the catalytically inactive mutant form of PTPμ induced LNCaP cell adhesion to purified recombinant PTPμ, demonstrating that PTPμ was indeed expressed at the cell surface at a level that could mediate homophilic binding. These results also show that perturbation of the phosphatase activity did not alter the subcellular localization or the ability of PTPμ to mediate homophilic binding as expected (4).

Although LNCaP cells express E-cadherin, as well as α-, β-, and γ-catenin and p120, they were unable to mediate E-cadherin-dependent adhesion. Re-expression of PTPμWT restored this adhesion, demonstrating a functional role for PTPμ in E-cadherin-mediated adhesion. The fact that the re-expression of the catalytically inactive mutant also restored E-cadherin-medi-

ated adhesion indicates that PTPμ exerts an effect on E-cadherin-dependent adhesion that is independent of its catalytic activity. This process requires the presence of the intracellular domain, because expression of PTPμ-extra failed to restore E-cadherin-mediated adhesion. It is possible that PTPμ alters the cadherin function by recruiting some signaling protein(s) to the cadherin complex through protein-protein interactions involving the PTPμ intracellular domain.

The interaction between RPTPs and various proteins may serve to regulate either the subcellular localization of RPTPs or to recruit other signaling molecules to form a larger signaling complex. The fact that PTPμ, regardless of its catalytic activity, could restore E-cadherin-mediated adhesion suggests that part of its role in the cadherin complex is to recruit other signaling molecules that may be needed for functional E-cadherin-dependent adhesion. The importance of the intracellular domain of PTPμ is clearly demonstrated by the finding that LNCaP adhesion to E-cadherin was not restored by the expression of a construct where the majority of the intracellular domain of PTPμ had been deleted (PTPμ-extra). In this regard, we isolated RACK1 as a protein that binds to the first phosphatase domain of PTPμ in a two-hybrid screen (18). RACK1 is a homologue of the Gβ subunit of heterotrimeric G-proteins (19) and consists of seven WD repeats that are believed to form a propeller-like structure (28). RACK1 is thought to be a scaffolding molecule because each of the seven WD repeats could potentially mediate protein-protein interactions. RACK-1 was originally described as a receptor for activated PKC (19), but more recent studies have described its interaction with a variety of signaling proteins, such as Src (29), and with select pleckstrin homology domains *in vitro* (30). In this study, we found that full-length PTPμ interacts with RACK1 and that this interaction is not dependent upon the catalytic activity of PTPμ. The interaction between PTPμ and RACK1 suggests that PTPμ may regulate E-cadherin-mediated adhesion by recruiting RACK1 and other signaling molecules to the PTPμ adhesion complex.

Despite numerous attempts to clarify the regulation of cadherin function by tyrosine phosphorylation, it is not fully understood. Tyrosine phosphorylation has been correlated with loss of cadherin-mediated adhesion and destabilization of adherens junctions (reviewed in Ref. 2). Therefore, adhesive function may be controlled by reversible tyrosine phosphorylation. Components of the cadherin-catenin complex are phosphorylated by a number of cytoplasmic and receptor protein tyrosine kinases including Src, EGF receptor, and Met (the scatter factor receptor) (2). In addition, PTPμ and a few other PTPs interact with cadherins and catenins (2). The association of the cadherins with both kinases and phosphatases indicates a critical role for dynamic tyrosine phosphorylation in cadherin function.

We performed studies on the role of tyrosine phosphorylation in regulating the association between PTPμ and E-cadherin in cells transformed with a temperature-sensitive form of the Rous sarcoma virus (14). The mutant Rous sarcoma virus is temperature-sensitive for $pp60^{src}$ tyrosine kinase activity. When grown at the permissive temperature, increased tyrosine phosphorylation induced by Src resulted in an increased tyrosine phosphorylation of E-cadherin, which correlated with a decreased association between PTPμ and E-cadherin. However, in this study we show that PTPμ regulates the cadherin function independently of its phosphatase activity, indicating that the cadherin-catenin complex may not be the primary substrates for PTPμ. We have shown previously that PTPμ catalytic activity is required for N-cadherin-mediated neurite outgrowth (16). These data indicate that PTPμ catalytic activity may be required for signaling events that regulate the cytoskeleton and thus other cadherin-dependent functions downstream of adhesion.

An alternative hypothesis is that the C-S mutant form of PTPμ may indirectly alter the tyrosine phosphorylation of the cadherin-catenin complex. In a recent paper (18), we found that PTPμ and Src compete for binding to RACK1. RACK1 binds to the SH2 domain of Src, an interaction that inhibits Src kinase activity (29). The interaction between RACK1 and PTPμ may regulate the presence of the Src protein tyrosine kinase in the cadherin-catenin complex. The presence of the PTPμ protein could recruit RACK1 to the plasma membrane where it could dissociate from PTPμ and possibly bind to and inactivate Src. Inactivation of Src could indirectly regulate the tyrosine phosphorylation of either E-cadherin or the catenins, thereby restoring E-cadherin-mediated adhesion.

Several studies have indicated that PKC is involved in the regulation of E-cadherin-mediated adhesion and the formation of adherens junctions. The molecular mechanisms whereby PKC regulates E-cadherin function are unknown. Additionally, the activation of PKC has been reported to have the opposite effects on E-cadherin function in different cell types. For example, the calcium-induced formation of adherens junctions in keratinocytes is dependent on the activation of PKC (24). On the other hand, activation of PKC has been shown to induce the dissociation of E-cadherin from the cytoskeleton (25), followed by cell scattering in the HT29 intestinal cell line (26). In this study, we show that the inhibition of PKCδ restored E-cadherin function in LNCaP cells. In addition, PTPμ restored E-cadherin-dependent adhesion, which could be reversed by PMA stimulation of PKCs. Together, these data suggest that PTPμ may negatively regulate PKC activity in LNCaP prostate carcinoma cells. Although the precise mechanism is unclear, it is likely to involve the PTPμ-RACK1 complex.

Others have shown (31, 32) that serine/threonine phosphorylation of p120 negatively regulates E-cadherin-mediated adhesion. It is therefore possible that the inactivation of PKCδ leads to decreased phosphorylation of p120 and thereby increased E-cadherin-mediated adhesion. However, we could not detect any alteration in the phosphorylation of p120 either after re-expression of PTPμ or after treatment of uninfected LNCaP cells with the PKCδ inhibitor rottlerin (data not shown). In addition, activation of PKC caused cells expressing PTPμWT to dissociate from an E-cadherin substrate. The fact that this dissociation occurred within 15 min after the addition of PMA argues that PKC directly affects the E-cadherin complex, rather than down-regulating the expression of either E-cadherin or the catenins. Therefore, the role of PTPμ in regulating E-cadherin-mediated adhesion could be to recruit RACK1 to the plasma membrane, thereby regulating the PKC pathway.

Acknowledgments—We thank Tracy Mourton, Leif Stordahl, Mary Chaiken, Polly Phillips-Mason, Sonya Ensslen, and Rachna Dave for providing excellent technical assistance. In addition, we thank Drs. Steven Reeves, Robert Brackenbury, Pamela Cowin, and Vance Lemmon for providing reagents. We also thank Dr. Carol Leidtke for reagents and helpful advice.

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